IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Constance A. Bell et al. Art Unit : 1637
Serial No. : 10/068,238 Examiner : Teresa E. Strzelecka
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Title : DETECTION OF BACILLUS ANTHRACIS

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Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

BRIEF ON APPEAL

(I) Real Party in Interest

The real party in interest is Mayo Foundation for Medical Education and Research.

(II) Related Appeals and Interferences

None.

(III) Status of Claims

Claims 57-96 are pending, and stand finally rejected.

(IV) Status of Amendments

All amendments have been entered.

(V) Summary of Claimed Subject Matter

In general, claims 57-96 are directed toward articles of manufacture that comprise two primers and/or two probes.

Independent claims 57, 59, 61, 63, 69, and 96 are directed toward articles of manufacture that comprise a pair of *capB* primers and/or a pair of *capB* probes. See, for example, page 12,

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lines 5-8. The pair of *capB* primers includes a first *capB* primer and a second *capB* primer (see, for example, page 12, lines 12-20), and the pair of *capB* probes includes a first *capB* probe and a second *capB* probe (see, for example, page 12, line 21 – page 13, line 6). The particular primer or probe sequences recited in claims 57-69 and 96 can be found, for example, in Example 1, pages 27-28.

Independent claims 70, 72, 74, 76, 82, and 96 are directed toward articles of manufacture that comprise a pair of *pagA* primers and/or a pair of *pagA* probes. See, for example, page 12, lines 5-8. The pair of *pagA* primers includes a first *pagA* primer and a second *pagA* primer (see, for example, page 12, lines 12-20), and the pair of *pagA* probes includes a first *pagA* probe and a second *pagA* probe (see, for example, page 12, line 21 – page 13, line 6). The particular primer or probe sequences recited in claims 70-82 and 96 can be found, for example, in Example 1, pages 27-28.

Specifically, independent claims 83, 85, 87, 89, 95, and 96 are directed toward articles of manufacture that comprise a pair of *lef* primers and/or a pair of *lef* probes. See, for example, page 12, lines 5-8. The pair of *lef* primers includes a first *lef* primer and a second *lef* primer (see, for example, page 12, lines 12-20), and the pair of *lef* probes includes a first *lef* probe and a second *lef* probe (see, for example, page 12, line 21 – page 13, line 6). The particular primer or probe sequences recited in claims 83-96 can be found, for example, in Example 1, pages 27-28.

(VI) Grounds of Rejection

Copies of the references are attached in Appendix IX.

(A) Claims 57-64 and 67-69 stand rejected under 35 U.S.C. §103 as being unpatentable over Ramisse et al. (*FEMS Microbiology Letters*, 145:9-16, 1996), Makino et al. (*J. Bacteriol.*, 171:722-30, 1989), and Buck et al. (*Biotechniques*, 1999, 27:528-36).

(B) Claims 65 and 66 stand rejected under 35 U.S.C. §103 as being unpatentable over Ramisse et al., Makino et al., Buck et al., and further in view of Wittwer et al. (*Biotechniques*, 22:130-138, 1997) and Qi et al. (*Appl. Env. Microbiol.*, 67:3720-3727, 2001).

(C) Claims 70-77 and 80-82 stand rejected under 35 U.S.C. §103 as being unpatentable over Ramisse et al., Price et al., and Buck et al.

(D) Claims 78 and 79 stand rejected under 35 U.S.C. §103 as being unpatentable over Ramisse et al., Price et al., Buck et al., and further in view of Wittwer et al. and Qi et al.

(E) Claims 83-90 and 93-95 stand rejected under 35 U.S.C. §103 as being unpatentable over Ramisse et al., Bragg et al., and Buck et al.

(F) Claims 91 and 92 stand rejected under 35 U.S.C. §103 as being unpatentable over Ramisse et al., Bragg et al., Buck et al., and further in view of Wittwer et al. and Qi et al.

(G) Claim 96 stands rejected under 35 U.S.C. §103 as being unpatentable over Ramisse et al., Makino et al., Price et al., Bragg et al., and Buck et al.

(VII) Arguments

(A) The 35 U.S.C. 103(a) rejections over Ramisse et al., Makino et al., and Buck et al.

Claims 57, 59, 61, 63, and 69

Ramisse et al. discloses *capB* primer sequences. Ramisse et al. does not teach or suggest the particular primer sequences recited in independent claims 57 (SEQ ID NO:1), 59 (SEQ ID NO:2), or 69 (SEQ ID NOs:1 and 2). Ramisse et al. also does not teach or suggest the particular probe sequences recited in independent claims 61 (SEQ ID NO:3), 63 (SEQ ID NO:4), or 69 (SEQ ID NOs:3 and 4).

Makino et al. discloses the sequence of *capB*. Makino et al. does not teach or suggest the particular primer sequences recited in independent claims 57, 59, or 69. Makino et al. also does not teach or suggest the particular probe sequences recited in independent claims 61, 63, or 69.

Buck et al. discloses that a number of different sequencing primers all were used successfully to sequence a particular target nucleic acid. Buck et al. compared sequencing primers using rhodamine or dichlororhodamine dye terminators in automated sequencing reactions. The results of Buck et al. were not based on amplification of *capB* nucleic acid sequences, and were not even based on amplification of *B. anthracis* nucleic acid sequences.

Even ignoring the fact that Buck et al. does not use *B. anthracis* nucleic acids, an automated sequencing reaction is significantly different than, for example, a PCR amplification reaction in which, generally, at least two oligonucleotides are used, or a real-time PCR amplification reaction in which, generally, at least four oligonucleotides are used. Applicants submit that the results reported by Buck et al. using sequencing primers are not representative of results using different primer and probe sequences in various types of amplification reactions. Primer design for PCR amplification and primer and probe design for real-time PCR

amplification is not always predictable. Applicants provide the following evidence to support this assertion.

The guidelines published by the University of Chicago Cancer Research Center DNA Sequencing Facility state "...be aware that no set of guidelines will always accurately predict the success of a primer. Some primers may fail for no apparent reason, and primers that appear to be poor candidates may work well." In addition, there are several peer-reviewed publications that compare different primer sets or compare the same primer set under different amplification conditions. For example, Csordas et al. (2004, *Lett. App. Microbiol.*, 39:187-193) states that "[p]rimers originally designed for end-point PCR did not have adequate specificity or sensitivity compared with those specifically designed for real-time PCR" (see the Abstract); Elnifro et al. (2000, *Clin. Microbiol. Rev.*, 13:559-570) states that "[e]mpirical testing and a trial-and-error approach may have to be used when testing several primer pairs, because there are no means to predict the performance characteristics of a selected primer pair even among those that satisfy the general parameters of primer design" (first full sentence on page 560); Tichopad et al. (2004, *Mol. Cell. Probes*, 18:45-50) states that "unknown tissue-specific factors can influence amplification kinetics but this affect can be ameliorated, in part, by appropriate primer selection" (see the Abstract); and Abd-Elsalam (2003, *African J. Biotech.*, 2:91-95) states that "...the most critical parameter for successful PCR is the design of primers" (see first full paragraph on page 94). See also Ballard et al., 2005, *Antimicrob. Agents Chemotherapy*, 49:77-81. Copies of these references are attached in Appendix IX.

The Examiner is using Buck et al. as a reference that ostensibly provides both motiviation and a reasonable expectation of success (i.e., that any oligonucleotide will work). Using a single oligonucleotide in a sequencing reaction as Buck et al. does is vastly different than using two, three, or four oligonucleotides in a real-time PCR amplification reaction. See, for example, Csordas et al., discussed above. The premise of Buck et al. may be true for sequencing reactions on the template DNAs reported therein, but it is certainly not true for amplification reactions, particularly real-time amplification reactions, on completely different template nucleic acids. Therefore, by combining various references with Buck et al., the Examiner has changed the principle of operation of Buck et al., which renders Buck et al. unsatisfactory for its purpose. See MPEP §2143.01, last section.

In addition, the particular primer and probe sequences recited in claims 57 (SEQ ID NO:1), 59 (SEQ ID NO:2), 61 (SEQ ID NO:3), 63 (SEQ ID NO:4), or 69 (SEQ ID NOs:1-4) are not obvious over any combination of the cited references because the sequences disclosed in the present specification exhibit high sensitivity and specificity toward their targets. Examples 1, 4 and 5 of the specification discuss the sensitivity and specificity of the claimed sequences. In addition, each of the claimed probe sequences (SEQ ID NOs:3 and 4) has a particular melting temperature, which can be used to confirm the presence or absence of *B. anthracis* in a sample. Therefore, the claimed probe sequences can be used to further increase the accuracy of detecting *B. anthracis*. See, for example, page 23, lines 5-7 and Example 3 of the specification.

The Examiner asserted that the claimed sequences represent "structural homologs" of the oligonucleotides taught in the prior art, and cited *In re Deuel* to support this assertion. *In re Deuel* is not relevant to the pending claims reciting a particular sequence, as *In re Deuel* dealt with the obviousness of nucleic acid sequences over prior art references that disclosed an amino acid sequence (or a partial amino acid sequence) encoded by such nucleic acid sequences. The claimed primer and probe sequences are not "structural homolog[s]" of the sequences disclosed in the art. *In re Deuel* does not indicate that a primer or probe sequence that is complementary to a target sequence is a "structural homolog."

Applicants are aware of no case law standing for the proposition that a longer sequence makes *per se* obvious specific primer and probe sequences from within that longer sequence. Based on the current case law, the claimed primer and probe sequences are not obvious over the cited references. See, for example, *In re Bell*, 991, F.2d 781, 26 USPQ2d 1529 (Fed. Cir. 1993) ("given the nearly infinite number of possibilities suggested by the prior art, and the failure of the cited prior art to suggest which of those possibilities [to select], the claimed sequences would not have been obvious"; Appendix IX).

In addition, a longer sequence can be viewed as representing a very large genus of possible sub-sequences from which appropriate primers and probes must be selected. Also based on current case law, the claimed primer and probe sequences are not obvious over the cited references. See, for example, *In re Jones*, 958 F.2d 347, 21 USPQ2d 1941 (Fed. Cir. 1992) ("[t]hat the claimed compound is a species of a genus disclosed in a prior art reference does not necessarily make the compound *prima facie* obvious"; Appendix IX).

Claims 58, 60, 62, and 64

Claim 58 depends from claim 57, and claim 60 depends from claim 59. Claims 58 and 60 each recite an article of manufacture that includes both the first *capB* primer sequence (SEQ ID NO:1) and the second *capB* primer sequence (SEQ ID NO:2). Claim 62 depends from claim 61, and claim 64 depends from claim 63. Claims 62 and 64 each recite an article of manufacture that includes both the first *capB* probe (SEQ ID NO:3) and the second *capB* probe (SEQ ID NO:4).

Applicants' arguments concerning deficiencies in the cited references, particularly those in Buck et al., and in the Examiner's reasoning, particularly with respect to *In re Deuel*, are reiterated. The cited references, in any combination, do not teach or suggest articles of manufacture that include the two particular primers recited in claims 58 and 60 or the two particular probes recited in claims 62 and 64.

Claims 67 and 68

Claim 67 depends from claims 57 or 59 and claim 68 depends from claims 61 or 63. Claims 67 and 68 are directed toward instructions for using the claimed *capB* primer sequences and *capB* probe sequences.

As indicated in the arguments above, independent claims 57, 59, 61, and 63 are not obvious over the cited references. According to MPEP §2143.03, if an independent claim is nonobvious under 35 USC §103, then any claim depending therefrom is nonobvious.

(B) The 35 U.S.C. §103(a) rejections over Ramisse et al., Makino et al., Buck et al., and further in view of Wittwer et al. and Qi et al.

Claims 65 and 66

Claims 65 and 66 depend, either directly or indirectly, from claims 61 or 63. Claim 65 is directed toward a donor fluorescent moiety and a corresponding acceptor fluorescent moiety, and claim 66 is directed toward the claimed *capB* probe sequences labeled with such fluorescent moieties.

Ramisse et al. is discussed above, and does not teach or suggest the probe sequences recited in independent claims 61 (SEQ ID NO:3) or 63 (SEQ ID NO:4).

Makino et al. is discussed above, and does not teach or suggest the probe sequences recited in independent claims 61 or 63.

Buck et al. is discussed above. The arguments above are reiterated with respect to this rejection and these claims.

Wittwer et al. discloses fluorescent donor-labeled and fluorescent acceptor-labeled probes. Wittwer et al. does not teach or suggest the probe sequences recited in independent claims 61 or 63.

Qi et al. discloses real-time PCR detection of *B. anthracis* using primers and probes directed toward *rpoB* nucleic acid sequences, wherein the probes are labeled with donor and acceptor moieties. Qi et al. does not teach or suggest the probe sequences recited in independent claims 61 or 63.

The remarks above regarding the high sensitivity and specificity of the claimed sequences and regarding the melting temperature are reiterated herein with respect to the probe sequences recited in independent claims 61 and 63.

The arguments regarding the Examiner's assertions with respect *In re Deuel* are reiterated with respect to this rejection and these claims.

(C) The 35 U.S.C. §103(a) rejections over Ramisse et al., Price et al., and Buck et al.

Claims 70, 72, 74, 76, and 82

Ramisse et al. discloses *pagA* primer sequences. Ramisse et al. does not teach or suggest the particular primer sequences recited in independent claims 70 (SEQ ID NO:5), 72 (SEQ ID NO:6), or 82 (SEQ ID NOs:5 and 6). Ramisse et al. also does not teach or suggest the particular probe sequences recited in independent claims 74 (SEQ ID NO:7), 76 (SEQ ID NO:8), or 82 (SEQ ID NOs:7 and 8).

Price et al. discloses *pagA* primers. Price et al. does not teach or suggest the particular primer sequences recited in independent claims 70, 72, or 82. Price et al. also does not teach or suggest the particular probe sequences recited in independent claims 74, 76, or 82.

Buck et al. is discussed above. The arguments above are reiterated with respect to this rejection and these claims.

The remarks above regarding the high sensitivity and specificity of the claimed primer and probe sequences are reiterated herein with respect to the primer and probe sequences recited in independent claims 70, 72, 74, 76, and 82.

The arguments regarding the Examiner's assertions with respect *In re Deuel* are reiterated with respect to this rejection and this claim.

Claims 71, 73, 75, and 77

Claim 71 depends from claim 70, and claim 73 depends from claim 72. Claims 71 and 73 each recite an article of manufacture that includes both the first *pagA* primer sequence (SEQ ID NO:5) and the second *pagA* primer sequence (SEQ ID NO:6). Claim 75 depends from claim 74, and claim 77 depends from claim 76. Claims 75 and 77 each recite an article of manufacture that includes both the first *pagA* probe (SEQ ID NO:7) and the second *pagA* probe (SEQ ID NO:8).

Applicants' arguments concerning deficiencies in the cited references, particularly those in Buck et al., and in the Examiner's reasoning, particularly with respect to *In re Deuel*, are reiterated. The cited references, in any combination, do not teach or suggest articles of manufacture that include the two particular primers recited in claims 71 and 73 or the two particular probes recited in claims 75 and 77.

Claims 80 and 81

Claim 80 depends from claims 70 or 72 and claim 81 depends from claims 74 or 76. Claims 80 and 81 are directed toward instructions for using the claimed *pagA* primer sequences and *pagA* probe sequences.

As indicated in the arguments above, independent claims 70, 72, 74, and 76 are not obvious over the cited references. According to MPEP §2143.03, if an independent claim is nonobvious under 35 USC §103, then any claim depending therefrom is nonobvious.

(D) The 35 U.S.C. §103(a) rejections over Ramisse et al., Price et al., Buck et al., and further in view of Wittwer et al. and Qi et al.

Claims 78 and 79

Claims 78 and 79 depend, either directly or indirectly, from claims 74 or 76. Claim 78 is directed toward a donor fluorescent moiety and a corresponding acceptor fluorescent moiety, and claim 79 is directed toward the claimed *pagA* probe sequences labeled with such fluorescent moieties.

Ramisse et al. is discussed above, and does not teach or suggest the probe sequences recited in independent claims 74 (SEQ ID NO:7) or 76 (SEQ ID NO:8).

Makino et al. is discussed above, and does not teach or suggest the probe sequences recited in independent claims 74 or 76.

Buck et al. is discussed above. The arguments above are reiterated with respect to this rejection and these claims.

Wittwer et al. is discussed above, and does not teach or suggest the probe sequences recited in independent claims 74 or 76.

Qi et al. is discussed above, and does not teach or suggest the probe sequences recited in independent claims 74 or 76.

The remarks above regarding the high sensitivity and specificity of the claimed sequences and regarding the melting temperature are reiterated herein with respect to the probe sequences recited in independent claims 74 and 76.

The arguments regarding the Examiner's assertions with respect *In re Deuel* are reiterated with respect to this rejection and these claims.

(E) The 35 U.S.C. §103(a) rejections over Ramisse et al., Bragg et al., and Buck et al.

Claims 83, 85, 87, 89, and 95

Ramisse et al. discloses *lef* primer sequences. Ramisse et al. does not teach or suggest the particular primer sequences recited in independent claims 83 (SEQ ID NO:9), 85 (SEQ ID NO:10), or 95 (SEQ ID NOs:9 and 10). Ramisse et al. also does not teach or suggest the particular probe sequences recited in independent claims 87 (SEQ ID NO:11), 89 (SEQ ID NO:12), or 95 (SEQ ID NOs:11 and 12).

Bragg et al. discloses the sequence of the *lef* gene. Bragg et al. does not teach or suggest the particular primer sequences recited in independent claims 83, 85, or 95. Bragg et al. also does not teach or suggest the particular probe sequences recited in independent claims 87, 89, or 95.

Buck et al. is discussed above. The arguments above are reiterated with respect to this rejection and these claims.

The remarks above regarding the high sensitivity and specificity of the claimed primer and probe sequences are reiterated herein with respect to the primer and probe sequences recited in independent claims 83, 85, 87, 89, and 95.

The arguments regarding the Examiner's assertions with respect *In re Deuel* are reiterated with respect to this rejection and these claims.

Claims 84, 86, 88, and 90

Claim 84 depends from claim 83, and claim 86 depends from claim 85. Claims 84 and 86 each recite an article of manufacture that includes both the first *lef* primer sequence (SEQ ID NO:9) and the second *lef* primer sequence (SEQ ID NO:10). Claim 88 depends from claim 87, and claim 90 depends from claim 89. Claims 88 and 90 each recite an article of manufacture that includes both the first *lef* probe (SEQ ID NO:11) and the second *lef* probe (SEQ ID NO:12).

Applicants' arguments concerning deficiencies in the cited references, particularly those in Buck et al., and in the Examiner's reasoning, particularly with respect to *In re Deuel*, are reiterated. The cited references, in any combination, do not teach or suggest articles of manufacture that include the two particular primers recited in claims 84 and 86 or the two particular probes recited in claims 88 and 90.

Claims 93 and 94

Claim 93 depends from claims 83 or 85 and claim 94 depends from claims 87 or 89. Claims 93 and 94 are directed toward instructions for using the claimed *lef* primer sequences and *lef* probe sequences.

As indicated in the arguments above, independent claims 83, 85, 87, and 89 are not obvious over the cited references. According to MPEP §2143.03, if an independent claim is nonobvious under 35 USC §103, then any claim depending therefrom is nonobvious.

(F) The 35 U.S.C. §103(a) rejections over Ramisse et al., Bragg et al., Buck et al., and further in view of Wittwer et al. and Qi et al.

Claims 91 and 92

Claims 91 and 92 depend, either directly or indirectly, from claims 87 or 89. Claim 91 is directed toward a donor fluorescent moiety and a corresponding acceptor fluorescent moiety, and claim 92 is directed toward the claimed *lef* probe sequences labeled with such fluorescent moieties.

Ramisse et al. is discussed above, and does not teach or suggest the probe sequences recited in independent claims 87 (SEQ ID NO:11) or 89 (SEQ ID NO:12).

Makino et al. is discussed above, and does not teach or suggest the probe sequences recited in independent claims 87 or 89.

Buck et al. is discussed above. The arguments above are reiterated with respect to this rejection and these claims.

Wittwer et al. is discussed above, and does not teach or suggest the probe sequences recited in independent claims 87 or 89.

Qi et al. is discussed above, and does not teach or suggest the probe sequences recited in independent claims 87 or 89.

The remarks above regarding the high sensitivity and specificity of the claimed sequences and regarding the melting temperature are reiterated herein with respect to the probe sequences recited in independent claims 87 and 89.

The arguments regarding the Examiner's assertions with respect *In re Deuel* are reiterated with respect to this rejection and these claims.

(G) The 35 U.S.C. §103(a) rejections over Ramisse et al., Makino et al., Price et al., Bragg et al., and Buck et al.

Claim 96

Claim 96 is directed toward an article of manufacture that includes all four *capB* primers and probes (SEQ ID NOs:1-4), all four *pagA* primers and probes (SEQ ID NOs:5-8), and all four *lef* primers and probes (SEQ ID NOs:9-12).

Ramisse et al. is discussed above. Ramisse et al. does not teach or suggest any of the primer or probe sequences recited in claim 96.

Makino et al. is discussed above. Makino et al. does not teach or suggest any of the primer or probe sequences recited in claim 96.

Price et al. is discussed above. Price et al. does not teach or suggest any of the primer or probe sequences recited in claim 96.

Bragg et al. is discussed above. Bragg et al. does not teach or suggest any of the primer or probe sequences recited in claim 96.

Buck et al. is discussed above. The arguments above are reiterated with respect to this rejection and these claims.

The remarks above regarding the high sensitivity and specificity of the claimed primer and probe sequences are reiterated herein with respect to the primer and probe sequences recited in claim 96.

The arguments regarding the Examiner's assertions with respect *In re Deuel* are reiterated with respect to this rejection and this claim.

Conclusions

Each pending independent claim recites a pair of primers and/or a pair of probes. Each independent claim recites the particular sequence of at least one primer or probe. These claimed sequences are not obvious over the known sequences in view of Buck et al. Therefore, the independent claims and those depending therefrom are not obvious over the cited references.

A check in the amount of $2,660 is enclosed ($500 for filing the Appeal Brief and $2,160 for the Extension of Time). Please apply any other charges or credits to Deposit Account No. 06-1050.

Respectfully submitted,

Date: July 19, 2005

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(VIII) Claims Appendix

1-57. (Canceled)

57. (Previously presented) An article of manufacture, comprising:

a pair of *capB* primers, wherein said pair of *capB* primers comprises a first *capB* primer and a second *capB* primer, wherein said first *capB* primer comprises the sequence 5'-CCC AAT TCG AGT AAA CAT A-3' (SEQ ID NO:1).

58. (Previously presented) The article of manufacture of claim 57, wherein said second *capB* primer comprises the sequence 5'-ACT GCC ATA CAT TCA CAA-3' (SEQ ID NO:2).

59. (Previously presented) An article of manufacture, comprising:

a pair of *capB* primers, wherein said pair of *capB* primers comprises a first *capB* primer and a second *capB* primer, wherein said second *capB* primer comprises the sequence 5'-ACT GCC ATA CAT TCA CAA-3' (SEQ ID NO:2).

60. (Previously presented) The article of manufacture of claim 59, wherein said first *capB* primer comprises the sequence 5'-CCC AAT TCG AGT AAA CAT A-3' (SEQ ID NO:1).

61. (Previously presented) An article of manufacture, comprising

a pair of *capB* probes, wherein said pair of *capB* probes comprises a first *capB* probe and a second *capB* probe, wherein said first *capB* probe comprises the sequence 5'-CGA TTA AGC GCC GTA AAG AAG GTC CTA ATA TC-3' (SEQ ID NO:3).

62. (Previously presented) The article of manufacture of claim 61, wherein said second *capB* probe comprises the sequence 5'-GTG AGC AAC GCA GGG TAG TTA AAG AGG CTG-3' (SEQ ID NO:4).

63. (Previously presented) An article of manufacture, comprising

a pair of *capB* probes, wherein said pair of *capB* probes comprises a first *capB* probe and a second *capB* probe, wherein said second *capB* probe comprises the sequence 5'-GTG AGC AAC GCA GGG TAG TTA AAG AGG CTG- 3' (SEQ ID NO:4).

64. (Previously presented) The article of manufacture of claim 63, wherein said first *capB* probe comprises the sequence 5'-CGA TTA AGC GCC GTA AAG AAG GTC CTA ATA TC-3' (SEQ ID NO:3).

65. (Previously presented) The article of manufacture of claim 61 or 63, further comprising a donor fluorescent moiety and a corresponding acceptor fluorescent moiety.

66. (Previously presented) The article of manufacture of claim 65, wherein said pair of *capB* probes comprises a first *capB* probe labeled with said donor fluorescent moiety and a second *capB* probe labeled with said corresponding acceptor fluorescent moiety.

67. (Previously presented) The article of manufacture of claim 57 or 59, further comprising a package label or package insert having instructions thereon for using said pair of *capB* primers to detect the presence or absence of *B. anthracis* in a biological sample.

68. (Previously presented) The article of manufacture of claim 61 or 63, further comprising a package label or package insert having instructions thereon for using said pair of *capB* probes to detect the presence or absence of *B. anthracis* in a biological sample.

69. (Previously presented) An article of manufacture comprising a pair of *capB* primers and a pair of *capB* probes, wherein said pair of *capB* primers comprises a first *capB* primer and a second *capB* primer, wherein said pair of *capB* probes comprises a first *capB* probe and a second *capB* probe, wherein said first *capB* primer comprises the sequence 5'-CCC AAT TCG AGT AAA CAT A-3' (SEQ ID NO:1), wherein said second *capB* primer comprises the sequence 5'-ACT GCC ATA CAT TCA CAA-3' (SEQ ID NO:2), wherein said first *capB* probe comprises the sequence 5'-CGA TTA AGC GCC GTA AAG AAG GTC CTA ATA TC-3' (SEQ ID NO:3), wherein said second *capB* probe comprises the sequence 5'-GTG AGC AAC GCA GGG TAG TTA AAG AGG CTG-3' (SEQ ID NO:4).

70. (Previously presented) An article of manufacture, comprising

a pair of *pagA* primers, wherein said pair of *pagA* primers comprises a first *pagA* primer and a second *pagA* primer, wherein said first *pagA* primer comprises the sequence 5'-TAC AGG ACG GAT TGA TAA G-3' (SEQ ID NO:5).

71. (Previously presented) The article of manufacture of claim 70, wherein said second *pagA* primer comprises the sequence 5'-TTT CAG CCC AAG TTC TTT-3' (SEQ ID NO:6).

72. (Previously presented) An article of manufacture, comprising

a pair of *pagA* primers, wherein said pair of *pagA* primers comprises a first *pagA* primer and a second *pagA* primer, wherein said second *pagA* primer comprises the sequence 5'-TTT CAG CCC AAG TTC TTT-3' (SEQ ID NO:6).

73. (Previously presented) The article of manufacture of claim 72, wherein said first *pagA* primer comprises the sequence 5'-TAC AGG ACG GAT TGA TAA G-3' (SEQ ID NO:5).

74. (Previously presented) An article of manufacture, comprising

a pair of *pagA* probes, wherein said pair of *pagA* probes comprises a first *pagA* probe and a second *pagA* probe, wherein said first *pagA* probe comprises the sequence 5'-AGT ACA TGG AAA TGC AGA AGT G- 3' (SEQ ID NO:7).

75. (Previously presented) The article of manufacture of claim 74, wherein said second *pagA* probe comprises the sequence 5'-ATG CGT CGT TCT TTG ATA TTG GT- 3' (SEQ ID NO:8).

76. (Previously presented) An article of manufacture, comprising

a pair of *pagA* probes, wherein said pair of *pagA* probes comprises a first *pagA* probe and a second *pagA* probe, wherein said second *pagA* probe comprises the sequence 5'-AGT ACA TGG AAA TGC AGA AGT G- 3' (SEQ ID NO:7).

77. (Previously presented) The article of manufacture of claim 76, wherein said first *pagA* probe comprises the sequence 5'-ATG CGT CGT TCT TTG ATA TTG GT- 3' (SEQ ID NO:8).

78. (Previously presented) The article of manufacture of claim 74 or 76, further comprising a donor fluorescent moiety and a corresponding acceptor fluorescent moiety.

79. (Previously presented) The article of manufacture of claim 78, wherein said pair of *pagA* probes comprises a first *pagA* probe labeled with said donor fluorescent moiety and a second *pagA* probe labeled with said corresponding acceptor fluorescent moiety.

80. (Previously presented) The article of manufacture of claim 70 or 72, further comprising a package label or package insert having instructions thereon for using said pair of *pagA* primers to detect the presence or absence of *B. anthracis* in a biological sample.

81. (Previously presented) The article of manufacture of claim 74 or 76, further comprising a package label or package insert having instructions thereon for using said pair of *pagA* probes to detect the presence or absence of *B. anthracis* in a biological sample.

82. (Previously presented) An article of manufacture comprising a pair of *pagA* primers and a pair of *pagA* probes, wherein said pair of *pagA* primers comprises a first *pagA* primer and a second *pagA* primer, wherein said pair of *pagA* probes comprises a first *pagA* probe and a second *pagA* probe, wherein said first *pagA* primer comprises the sequence 5'-TAC AGG ACG GAT TGA TAA G-3' (SEQ ID NO:5), wherein said second *pagA* primer comprises the sequence 5'-TTT CAG CCC AAG TTC TTT-3' (SEQ ID NO:6), wherein said first *pagA* probe comprises the sequence 5'-AGT ACA TGG AAA TGC AGA AGT G- 3' (SEQ ID NO:7), wherein said second

pagA probe comprises the sequence 5'-ATG CGT CGT TCT TTG ATA TTG GT- 3' (SEQ ID NO:8).

83. (Previously presented) An article of manufacture, comprising

a pair of *lef* primers, wherein said pair of *lef* primers comprises a first *lef* primer and a second *lef* primer, wherein said first *lef* primer comprises the sequence 5'-TTT TAC CGA TAT TAC TCT CC-3' (SEQ ID NO:9).

84. (Previously presented) The article of manufacture of claim 83, wherein said second *lef* primer comprises the sequence 5'-AAC CTA AAG GCT TCT GC-3' (SEQ ID NO:10).

85. (Previously presented) An article of manufacture, comprising

a pair of *lef* primers, wherein said pair of *lef* primers comprises a first *lef* primer and a second *lef* primer, wherein said second *lef* primer comprises the sequence 5'-AAC CTA AAG GCT TCT GC-3' (SEQ ID NO:10).

86. (Previously presented) The article of manufacture of claim 85, wherein said first *lef* primer comprises the sequence 5'-TTT TAC CGA TAT TAC TCT CC-3' (SEQ ID NO:9).

87. (Previously presented) An article of manufacture, comprising

a pair of *lef* probes, wherein said pair of *lef* probes comprises a first *lef* probe and a second *lef* probe, wherein said first *lef* probe comprises the sequence 5'-ATT AAG GAA TGA TAG TGA GGG T- 3' (SEQ ID NO:11).

88. (Previously presented) The article of manufacture of claim 87, wherein said second *lef* probe comprises the sequence 5'-TAT ACA CGA ATT TGG ACA TGC T- 3' (SEQ ID NO:12).

89. (Previously presented) An article of manufacture, comprising

a pair of *lef* probes, wherein said pair of *lef* probes comprises a first *lef* probe and a second *lef* probe, wherein said second *lef* probe comprises the sequence 5'-TAT ACA CGA ATT TGG ACA TGC T- 3' (SEQ ID NO:12).

90. (Previously presented) The article of manufacture of claim 89, wherein said first *lef* probe comprises the sequence 5'-ATT AAG GAA TGA TAG TGA GGG T- 3' (SEQ ID NO:11).

91. (Previously presented) The article of manufacture of claim 87or 89, further comprising a donor fluorescent moiety and a corresponding acceptor fluorescent moiety.

92. (Previously presented) The article of manufacture of claim 91, wherein said pair of *lef* probes comprises a first *lef* probe labeled with said donor fluorescent moiety and a second *lef* probe labeled with said corresponding acceptor fluorescent moiety.

93. (Previously presented) The article of manufacture of claim 83 or 85, further comprising a package label or package insert having instructions thereon for using said pair of *lef* primers to detect the presence or absence of *B. anthracis* in a biological sample.

94. (Previously presented) The article of manufacture of claim 87 or 89, further comprising a package label or package insert having instructions thereon for using said pair of *lef* probes to detect the presence or absence of *B. anthracis* in a biological sample.

95. (Previously presented) An article of manufacture comprising a pair of *lef* primers and a pair of *lef* probes, wherein said pair of *lef* primers comprises a first *lef* primer and a second *lef* primer, wherein said pair of *lef* probes comprises a first *lef* probe and a second *lef* probe, wherein said first *lef* primer comprises the sequence 5'-TTT TAC CGA TAT TAC TCT CC-3' (SEQ ID NO:9), wherein said second *lef* primer comprises the sequence 5'-AAC CTA AAG GCT TCT GC-3' (SEQ ID NO:10), wherein said first *lef* probe comprises the sequence 5'-ATT AAG GAA TGA TAG TGA GGG T- 3' (SEQ ID NO:11), wherein said second *lef* probe comprises the sequence 5'-TAT ACA CGA ATT TGG ACA TGC T- 3' (SEQ ID NO:12).

96. (Previously presented) An article of manufacture comprising a pair of *capB* primers and a pair of *capB* probes, wherein said pair of *capB* primers comprises a first *capB* primer and a second *capB* primer, wherein said pair of *capB* probes comprises a first *capB* probe and a second *capB* probe, wherein said first *capB* primer comprises the sequence 5'-CCC AAT TCG AGT AAA CAT A-3' (SEQ ID NO:1), wherein said second *capB* primer comprises the sequence 5'-ACT GCC ATA CAT TCA CAA-3' (SEQ ID NO:2), wherein said first *capB* probe comprises the sequence 5'-CGA TTA AGC GCC GTA AAG AAG GTC CTA ATA TC-3' (SEQ ID NO:3), wherein said second *capB* probe comprises the sequence 5'-GTG AGC AAC GCA GGG TAG TTA AAG AGG CTG-3' (SEQ ID NO:4), said article of manufacture further comprising a pair of *pagA* primers and a pair of *pagA* probes, wherein said pair of *pagA* primers comprises a first *pagA* primer and a second *pagA* primer, wherein said pair of *pagA* probes comprises a first *pagA* probe and a second *pagA* probe, wherein said first *pagA* primer comprises the sequence 5'-TAC AGG ACG GAT TGA TAA G-3' (SEQ ID NO:5), wherein said second *pagA* primer comprises the sequence 5'-TTT CAG CCC AAG TTC TTT-3' (SEQ ID NO:6), wherein said first *pagA*

probe comprises the sequence 5'-AGT ACA TGG AAA TGC AGA AGT G- 3' (SEQ ID NO:7), wherein said second *pagA* probe comprises the sequence 5'-ATG CGT CGT TCT TTG ATA TTG GT- 3' (SEQ ID NO:8), said article of manufacture further comprising a pair of *lef* primers and a pair of *lef* probes, wherein said pair of *lef* primers comprises a first *lef* primer and a second *lef* primer, wherein said pair of *lef* probes comprises a first *lef* probe and a second *lef* probe, wherein said first *lef* primer comprises the sequence 5'-TTT TAC CGA TAT TAC TCT CC-3' (SEQ ID NO:9), wherein said second *lef* primer comprises the sequence 5'-AAC CTA AAG GCT TCT GC-3' (SEQ ID NO:10), wherein said first *lef* probe comprises the sequence 5'-ATT AAG GAA TGA TAG TGA GGG T- 3' (SEQ ID NO:11), wherein said second *lef* probe comprises the sequence 5'-TAT ACA CGA ATT TGG ACA TGC T- 3' (SEQ ID NO:12).

(IX) Evidence Appendix

The following are attached herewith.

(A) Ramisse et al., *FEMS Microbiology Letters*, 145:9-16, 1996;

(B) Makino et al., *J. Bacteriol.*, 171:722-30, 1989;

(C) Buck et al., *Biotechniques*, 1999, 27:528-36;

(D) Wittwer et al., *Biotechniques*, 22:130-138, 1997;

(E) Qi et al., *Appl. Env. Microbiol.*, 67:3720-3727, 2001;

(F) Csordas et al., 2004, *Lett. App. Microbiol.*, 39:187-193;

(G) Elnifro et al., 2000, *Clin. Microbiol. Rev.*, 13:559-570;

(H) Tichopad et al., 2004, *Mol. Cell. Probes*, 18:45-50;

(I) Abd-Elsalam, 2003, *African J. Biotech.*, 2:91-95;

(J) Ballard et al., 2005, *Antimicrob. Agents Chemotherapy*, 49:77-81;

(K) *In re Deuel*, 34 USPQ 2d (Fed. Cir. 1995);

(L) *In re Bell*, 991, F.2d 781, 26 USPQ2d 1529 (Fed. Cir. 1993); and

(M) *In re Jones*, 958 F.2d 347, 21 USPQ2d 1941 (Fed. Cir. 1992).

(X) Related Proceedings Appendix

None

FEMS Microbiology Letters 145 (1996) 9–16

FEMS MICROBIOLOGY LETTERS

Identification and characterization of *Bacillus anthracis* by multiplex PCR analysis of sequences on plasmids pXO1 and pXO2 and chromosomal DNA

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Abstract

Bacillus anthracis can be identified on the basis of the detection of virulence factor genes located on two plasmids, pXO1 and pXO2. Thus isolates lacking both pXO1 and pXO2 are indistinguishable from closely related *B. cereus* group bacteria. We developed a multiplex PCR assay for characterization of *B. anthracis* isolates, and simultaneous confirmation of the species identity independent of plasmid content. The assay amplifies *lef*, *cya*, *pag* (pXO1) and *cap* (pXO2) genes, and a *B. anthracis* specific chromosomal marker, giving an easy-to-read profile. This system unambiguously identified virulent ($pXO1^+/2^+$) and avirulent ($pXO1^+/2^-$, $pXO1^-/2^+$ and $pXO1^-/2^-$) strains of *B. anthracis* and distinguished 'anthrax-like' strains from other *B. cereus* group bacteria.

Keywords: Bacillaceae; *Bacillus anthracis*; *Bacillus cereus*; *Bacillus anthracis* genetics; Anthrax epidemiology; Bacterial identification; Diagnosis; Polymerase chain reaction

1. Introduction

Bacillus anthracis is a human pathogenic bacillus. It can cause anthrax, a serious and often fatal infection in both livestock and humans. Animals become infected after coming into contact with soil-borne spores when grazing. Humans become infected only incidentally when brought into contact with diseased animals or their waste products. Other *Bacillus* species are saprophytic and are found in soil, vegetation, air and water and some species such as *B. cereus* are responsible for benign food poisoning [1].

The current laboratory diagnosis for *B. anthracis* is the microbiological analysis of morphological and physiological characteristics. The main characteristic used to distinguish *B. anthracis* from closely related soil-borne *Bacillus* is the presence of two virulence

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Table 1
Strains tested and genotyping of *B. anthracis* isolates examined

Name	CEB no.	Original ID	Plasmid content	Single PCR results (58°C)				
				lef 3–4	*cya* 25–26	*pag* 67–68	*cap* 57–58	Ba813 R1–R2
Clostridium botulinum A	94-7	CEB	na	–	–	–	–	–
Yersinia pestis	na	31-93	na	–	–	–	–	–
Yersinia enterocolitica	85-15	CEB	na	–	–	–	–	–
Streptococcus pyogenes A	90-6	CEB	na	–	–	–	–	–
Staphylococcus aureus	90-1	CovanIII	na	–	–	–	–	–
Escherichia coli	85-35	CEB	na	–	–	–	–	–
Citrobacter freundii	86-45	CEB	na	–	–	–	–	–
Enterobacter aerogenes	86-46	CEB	na	–	–	–	–	–
Proteus mirabilis	86-51	CEB	na	–	–	–	–	–
Providencia rettgeri	86-52	CEB	na	–	–	–	–	–
Shigella boydii	86-58	CEB	na	–	–	–	–	–
Salmonella arizonae	87-12	CEB	na	–	–	–	–	–
Pseudomonas aeruginosa	90-27	CEB	na	–	–	–	–	–

Name	CEB no.	Original ID	Plasmid content	Multiplex PCR results (58°C)				
				lef 3–4	*cya* 25–26	*pag* 67–68	*cap* 57–58	Ba813 R1–R2
Bacillus mycoides	96-12	ATCC 6462	na	–	–	–	–	–
Bacillus cereus	92-11	ATCC 14579	na	–	–	–	–	–
Bacillus thuringiensis	92-6	ATCC 10792	na	–	–	–	–	–
Bacillus licheniformis	92-4	ATCC 14580	na	–	–	–	–	–
Bacillus stearothermophilus	92-5	ATCC 7953	na	–	–	–	–	–
Bacillus sphaericus	92-7	ATCC 14577	na	–	–	–	–	–
Bacillus alvei	92-8	ATCC 6344	na	–	–	–	–	–
Bacillus megaterium	92-9	ATCC 14581	na	–	–	–	–	–
Bacillus pasteurii	92-10	ATCC 11859	na	–	–	–	–	–
Bacillus pumilus	92-3	ATCC 7051	na	–	–	–	–	–
Bacillus subtilis	92-2	ATCC 6051	na	–	–	–	–	–
Bacillus subtilis	92-12	ATCC 9372	na	–	–	–	–	+
Bacillus anthracis	95-31	Sterne 7700	$pXO1^-/2^-$	+	+	+	–	+
Bacillus anthracis	95-32	Sterne 7702	$pXO1^+/2^-$	–	–	–	+	+
Bacillus anthracis	95-36	ATCC 4229	$pXO1^-/2^+$	–	–	–	+	+
Bacillus anthracis	95-33	ATCC 6602	$pXO1^-/2^+$	+	+	+	–	+
Bacillus anthracis	96-6	Cepanzo	$pXO1^+/2^-$	–	–	–	+	+
Bacillus anthracis	95-34	Davis TE 702	$pXO1^-/2^+$	+	+	+	+	+
Bacillus anthracis	227	This study	$pXO1^-/2^+$	+	+	+	–	+
Bacillus anthracis	957	This study	$pXO1^+/2^-$	+	+	+	+	+
Bacillus anthracis	170	This study	$pXO1^+/2^+$	+	+	+	+	+
Bacillus anthracis	300	This study	$pXO1^+/2^+$	+	+	+	+	+
Bacillus anthracis	779	This study	$pXO1^+/2^+$	+	+	+	+	+
Bacillus anthracis	832	This study	$pXO1^+/2^+$	+	+	+	+	+
Bacillus anthracis	663	This study	$pXO1^-/2^+$	+	+	+	+	+
Bacillus anthracis	376	This study	$pXO1^-/2^+$	+	+	+	+	+
Bacillus anthracis	846	This study	$pXO1^+/2^+$	+	+	+	+	+
Bacillus anthracis	256	This study	$pXO1^+/2^+$	+	+	+	+	+
Bacillus anthracis	582	This study	$pXO1^+/2^+$	+	+	+	+	+
Bacillus anthracis	282	This study	$pXO1^+/2^+$	+	+	+	+	+
Bacillus anthracis	85	This study	$pXO1^+/2^+$	+	+	+	+	+
Bacillus anthracis	576	This study	$pXO1^+/2^+$	+	+	+	+	+
Bacillus anthracis	955	This study	$pXO1^+/2^+$	+	+	+	+	+

Table 1 (continued)

Name	CEB no.	Original ID	Plasmid content	Single PCR results (58°C)				
				lef 3–4	*cya* 25–26	*pag* 67–68	*cap* 57–58	Ba813 R1–R2
Bacillus anthracis	554	This study	pXO1⁻/2⁻	–	–	–	–	+
Bacillus anthracis	657	This study	pXO1⁻/2⁺	+	+	+	+	+
Bacillus anthracis	516	This study	pXO1⁺/2⁺	+	+	+	+	+
Bacillus anthracis	346	This study	pXO1⁺/2⁺	+	+	+	+	+
Bacillus anthracis	552	This study	pXO1⁺/2⁺	+	+	+	+	+
Bacillus anthracis	470	This study	pXO1⁻/2⁻	+	+	+	+	+
Bacillus anthracis	575	This study	pXO1⁺/2⁻	+	+	+	–	+
Bacillus anthracis	204	This study	pXO1⁻/2⁺	+	+	+	+	+
Bacillus anthracis	893	This study	pXO1⁺/2⁺	+	+	+	+	+
Bacillus anthracis	69	This study	pXO1⁻/2⁻	+	+	+	–	+

na, not applicable.
[a]See text for details.

plasmids pXO1 (185 kb) and pXO2 (95 kb). Virulence factor genes *lef*, *cya*, *pag* (pXO1) [2–4] or *cap* (pXO2) [5] have been used as markers to detect *B. anthracis* in the environment using the polymerase chain reaction (PCR) [6–8].

pXO1 and pXO2-cured strains have arisen spontaneously from fully virulent precursors [9]. These avirulent strains cannot be distinguished from other *B. cereus* group bacteria by plasmid analysis. Other conventional methods including gamma phage sensitivity test or carbohydrate profile have to be used. DNA homology studies [10] have shown that *B. anthracis* is very closely related to *B. cereus*, *B. thuringiensis* and *B. mycoides*. The taxonomy of this group is controversial because of the many similarities between the nucleotide sequences of 16S rRNA and 23S rRNA genes, and 16S to 23S intergenic spacer region in the various isolates [11–13]. Nevertheless, genetic variability among *B. anthracis* and other *B. cereus* group bacteria has been reported [14–17]. As an example, the variable region with repetitive sequences (*vrr*A) diverged only slightly between strains of anthrax but is substantially different in *B. cereus* and *B. mycoides* [16]. More recently, a DNA sequence (Ba813) isolated from *B. anthracis* was described as a specific chromosomal marker for this species [17]. Ba813 is a 277 bp long DNA sequence and is present in a single copy in the chromosome of *B. anthracis*. Nucleotide sequence and deduced amino acid sequence comparisons with previously described sequences revealed no homology [17], thus providing a simple way to differentiate *B. anthracis* from the *B. cereus* group.

The increasingly frequent isolation of avirulent *B. anthracis* from humans and animals [9,18] is creating a need for a rapid identification system. The purpose of this study was to develop a PCR-based identification system for *B. anthracis* with the following characteristics: (i) screening for the presence of pXO1 and pXO2, (ii) identifying all known virulence factors (*lef*, *cya*, *pag* and *cap*) (iii) differentiating *B. anthracis* from other *B. cereus* group bacteria. We report a multiplex PCR assay that should be convenient for most clinical and veterinary laboratories and reliable for plasmid content comparisons between strains.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Bacterial strains used in this study are listed in Table 1. *B. anthracis* isolates were obtained from various collections in France. Bacilli were cultured as previously described [17]. Other strains were cultured in soy-trypticase broth (BioMérieux).

2.2. DNA preparation

Genomic DNA from *B. anthracis* bacteria was isolated from pure cultures by standard procedures [17].

Control DNAs were extracted from the various species listed in Table 1 [17]. Genomic DNA from *Yersinia pestis* (a strain bearing pFra, pYV and pPst plasmids) was kindly provided by Dr. Elisabeth Carniel (Institut Pasteur, Paris).

2.3. Oligonucleotides

Oligonucleotides R1 and R2 were synthesised previously to amplify by PCR a 152 bp fragment from the sequence Ba813, at 59°C [17]. Two primer pairs were designed from *lef*, *cya*, *pag*, and *cap* sequences [2–5] by computer analysis using the Oligo® primer analysis software (MedProbe, Oslo, Norway). The sequences for these primers were designed to amplify all virulence factor genes and the Ba813 marker using the following guidelines: (i) the annealing temperature should be close to 59°C; (ii) the length of amplification products should be between 1 kbp and 200 bp (Table 2).

2.4. DNA amplification procedure

Each 25 μl reaction mixture contained 200 μM of each dATP, dCTP, dGTP and dTTP, 0.5 μM of primers, 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 13% (w/v) sucrose, 40 mg ml^{-1} cresol red, 1 U of *Taq*-DNA polymerase (Boehringer) and 100 ng of DNA. Amplifications were carried out in a GeneAmp® PCR system 9600 (Perkin Elmer). The initial denaturation at 94°C for 5 min was followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s. The reaction mixtures (10 μl) were directly loaded onto 2% (w/v) agarose (Sea-Kem GTG, FMC Bioproducts) gel and DNA fragments separated at 100 V for 1 h before staining with ethidium bromide.

2.5. Hybridization techniques

PCR products were purified using the Sephaglass band prep kit (Pharmacia) and labelled with [α-^{32}P]dCTP (ICN) and the random priming labelling kit (Boehringer). Dot-blot hybridization was at

Table 2
Sequence and position of the primers used in the polymerase chain reaction

Primers	Loci	Positions[a]	T_m (°C)[b]	T_p (°C)[c]	Length (bp)	Sequence (5′→3′)	Reference
67	*pag*	1925–1944	63	64	747	CAGAATCAAGTTCCCAGGGG	This study
68		2652–2671	63			TCGGATAAGCTGCCACAAGG	
23	*pag*	2006–2027	59	58	151	CTACAGGGGATTTATCTATTCC	This study
24		2135–2156	57			ATTGTTACATGATTATCAGCGG	
65	*cya*	1255–1274	58	60	929	CAGCATGCGTTTTCTTTAGC	This study
66		2164–2183	58			CCCTTAGTTGAATCCGGTTT	
25	*cya*	1459–1478	58	59	546	GGTTTAGTACCAGAACATGC	This study
26		1990–2004	61			CGGCTTCAAGACCCC	
59	*lef*	949– 970	54	60	993	GGATATGAACCCGTACTTGTAA	This study
60		1921–1941	62			TAAATCCGCACCTAGGGTTGC	
3	*lef*	1238–1258	54	54	385	CTTTTGCATATTATATCGAGC	This study
4		1599–1622	57			GAATCACGAATATCAATTTGTAGC	
17	*cap*	1230–1249	54	57	873	GAAATAGTTATTGCGATTGG	This study
20	(B, C, A)	2083–2102	63			GGTGCTACTGCTTCTGTACG	
57	*cap* (C)	1603–1622	58	57	264	ACTCGTTTTTAATCAGCCCG	This study
58		1847–1866	56			GGTAACCCTTGTCTTTGAAT	
R1	Ba813	227– 249	59	58	152	TTAATTCACTTGCAACTGATGGG	[17]
R2		98– 120	60			AACGATAGCTCCTACATTTGGAG	

[a]Positions are given according to published sequences [2–5].
[b]Primer T_m was calculated by the nearest-neighbor method [19].
[c]Optimized annealing temperature.

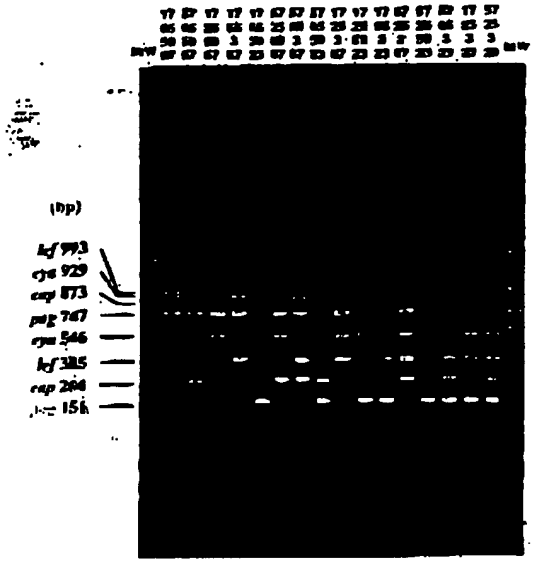

Fig. 1. Simultaneous PCR-based amplification of capsule (*cap*), edema factor (*cya*), lethal factor (*lef*) and protective antigen (*pag*) genes from 100 ng of DNA from the virulent strain of *Bacillus anthracis* CEB779. Primers combinations are indicated on the top: forward primer only shown. MW: *Sau*3A and *Taq*I digested pUC19 DNA size markers: 1444, 955, 735, 584, 476, 341, 258 and 141 bp.

65°C and membranes were washed at low stringency (2SSC, 65°C) before exposure.

3. Results and discussion

3.1. Specificity of oligonucleotide primers

We attempted to amplify the targeted fragments from 100 ng samples of DNA isolated from Sterne 7700, Sterne 7702 and ATCC4229 strains of *B. anthracis*. Fragments of the expected size were successfully amplified from DNA of all appropriate strains of *B. anthracis* (data not shown). The annealing temperature was optimized for each primer pair and the results are given in Table 2. The specificity of oligonucleotide primers was checked by PCR with 100 ng of DNA from other *Bacillus* species and strains belonging to other genera (Table 1). No amplified products were generated from these control DNAs with one exception: small amounts of products of incorrect size were amplified from *Y. pestis* (a major product of 700 bp and four minor products of between 200 bp and 3 kbp) and from *Y. enterocolitica* (a major product of 950 bp and four minor products of between 500 bp and 1.5 kbp) with primers 25–26 (data not shown). The 546 bp *pag* fragment amplified from *B. anthracis* and the above non-specific major fragments were labeled and tested for hybridization with genomic DNA (1 μg) from *B. anthracis*, *Y. pestis* and *Y. enterocolitica*. Therefore, the nucleotide sequences of these PCR products are non-homologous. Each probe only hybridized with the DNA from which it was amplified (data not shown). We hypothesize that the DNA of *Y. pestis* carries sequences of partial homology allowing hybridization of primers 25 and/or 26.

3.2. Multiplex PCR assays

In preliminary experiments using mixtures of primer pairs neither the number nor the size of DNA fragments was modified (unpublished data). We tested whether simultaneous amplification of *lef*, *cya*, *pag* and *cap* was feasible. Primer sets from Table 2 were pooled so that all of the four targeted genes would be amplified once per assay (16 combinations tested consisting of 8 sets). DNA fragments with the expected size were successfully amplified using nearly all combinations (Fig. 1). However, a minor amplified product of about 500 bp is present in all experiments involving the primer pair 3/4. To this point we noted that: (i) single amplification using only the primer pair 3/4 did not generate this fragment, and neither did single amplifications using primer 3 or 4 separately; (ii) the amplification of the 385 bp DNA fragment (3/4) is significantly hampered in multiplex conditions compared to single condition (data not shown). From that, we hypothesize that an illegitimate primer pair, involving primer 3 or 4 and another one present in the mixture, may occur in multiplex conditions, thus allowing a slight amplification of the 500 bp fragment from the genome of *B. anthracis*. The combination of primer pairs 67–68, 25–26, 3–4 and 57–58 primer pairs was the most reliable one in terms of amplification efficiency and gave easily recognizable patterns, thus making DNA fragment profiles easy to interpret for plasmid composi-

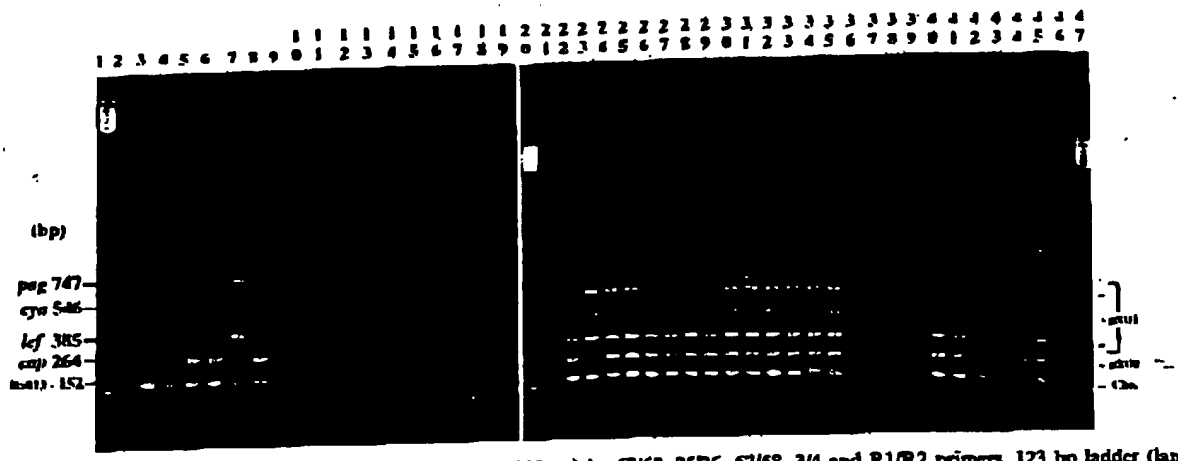

Fig. 2. Results of the amplification of total genomic DNA (~100 ng) by 67/68, 25/26, 57/58, 3/4 and R1/R2 primers. 123 bp ladder (lanes 1,20,47), negative control (lanes 2,21). *B. anthracis* Sterne 7700 (lane 3), Sterne 7702 (lane 4), pXO1$^-$/2$^+$ ATCC4429 (lane 5), pXO1$^-$/2$^+$ ATCC6602 (lane 6), Cepanzo (lane 7), Davis TE702 (lane 8), *B. subtilis* ATCC6051 (lane 9), *B. pumilus* ATCC7061 (lane 10), *B. licheniformis* ATCC14580 (lane 11), *B. stearothermophilus* ATCC7953 (lane 12), *B. thuringiensis* ATCC10792 (lane 13), *B. sphaericus* ATCC14577 (lane 14), *B. alvei* ATCC6344 (lane 15), *B. megaterium* ATCC14581 (lane 16), *B. pasteurii* ATCC11859 (lane 17), *B. cereus* ATCC14579 (lane 18), *B. subtilis* ATCC9372 (lane 19), *B. anthracis* 227 (lane 22), 957 (lane 23), 170 (lane 24), 300 (lane 25), 779 (lane 26), 832 (lane 27), 663 (lane 28), 376 (lane 29), 846 (lane 30), 256 (lane 31), 582 (lane 32), 282 (lane 33), 85 (lane 34), 576 (lane 35), 955 (lane 36), 554 (lane 37), 657 (lane 38), 516 (lane 39), 346 (lane 40), 552 (lane 41), 470 (lane 42), 575 (lane 43), 204 (lane 44), 893 (lane 45) and 69 (lane 46).

tion. Single PCR, targeting virulence factor genes one by one, has been used to characterize *B. anthracis* isolates [9]. Our system, by targeting several virulence factor genes together in the same reaction mixture, makes plasmid content analysis of pure cultures of *B. anthracis* much simpler.

This multiplex PCR assay was not suitable for general identification of *B. anthracis* because a strain lacking both pXO1 and pXO2 would not be identified. Thus, a multiplex PCR using 67–68, 25–26, 3–4, 57–58 (plasmid markers) and R1–R2 primers (chromosomal marker) was tested under the same conditions. Amplification of pXO1 and pXO2 associated virulence factors was still efficient despite the presence of a fifth primer set. The 152 bp DNA fragment from the Ba813 sequence was also efficiently amplified. The specificity of the PCR assay using these five primer pairs was tested against 31 *B. anthracis* isolates and 12 *Bacillus* strains from 11 other species (Fig. 2). The 152-bp DNA fragment was successfully amplified from each of the 31 strains of *B. anthracis* tested but not from other *Bacillus* species, thus confirming species identity. No extra PCR products were generated from closely related members of the *B. cereus* group (Fig. 2). Six well known avirulent strains and 25 isolates of *B. anthracis* were then successfully screened for plasmid content. Each possible type of *B. anthracis* was correctly identified (pXO1$^+$/2$^+$, pXO1$^+$/2$^-$, pXO1$^-$/2$^+$, pXO1$^-$/2$^-$). Three isolates of *B. anthracis* were characterized as pXO1$^+$/2$^-$ (CEB 957, 575 and 69), one as pXO1$^-$/2$^-$ (CEB 554). The others were characterized as fully virulent (pXO1$^+$/2$^+$). Specialized laboratories involved in anthrax control and research use elaborate techniques for confirming strain identity: toxin antigen detection [9], detection of pXO1 and pXO2 virulence factor genes by hybridization with DNA and oligonucleotide probes [7,9,20] or by PCR [8,9]. No single existing method is able to screen for plasmid-associated virulence factor genes and simultaneously to differentiate *B. anthracis* (whether or not containing plasmids) from other *Bacillus* species. Our multiplex PCR assay may meet these requirements and is rapid: less than 3 h after picking an isolated colony from an agar plate.

In summary, the method described here is the first

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References

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example of a rapid procedure enabling identification and genotyping of *B. anthracis*, and simultaneous differentiation from other *B. cereus* group bacteria. Identification of *B. anthracis*, independent of the plasmid content is practicable using the chromosomal Ba813 sequence reported by Patra et al. [17] and the outstanding characterization of the whole marker on the both sides of Ba813 sequence will provide more detailed information. *B. anthracis* isolates that carry neither pXO1 nor pXO2 are clearly distinguished from other *Bacillus* species thus enabling plasmid-free isolates and vaccine seed cultures of *B. anthracis* to be followed. Our method also reduces the identification time from the standard 3–6 days to less than 2 days. This powerful methodology will contribute to reducing the risk of a laboratory-acquired infection and should be of great help as a routine test for confirmation of *B. anthracis* in clinical and environmental samples.

Acknowledgments

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References

[1] Gilbert, R.J., Turbull, P.C.B., Parry, J.M. and Kramer, J.M. (1981) *Bacillus cereus* and other *Bacillus* species: their part in food poisoning and other clinical infections. In: The Aerobic Endospore-forming Bacteria: Classification and Identification (Berkeley, R.C.W. and Goodfellow, M., Eds.), pp. 295–314, Academic Press, London.

[2] Bragg, T.S. and Robertson, D.L., (1989) Nucleotide sequence of the lethal factor gene from *Bacillus anthracis*. Gene 81, 45–54.

[3] Escuyer, V., Duflot, E., Sezer, O., Danchin, A. and Mock, M. (1988) Structural homology between virulence-associated bacterial adenylate cyclase. Gene 71, 293–298.

[4] Welkos, S.L., Lowe, J.R., Eden-McCutchan, F., Vodkin, M., Leppla, S.H. and Schmidt, J.J. (1988) Sequence and analysis of the DNA encoding protective antigen of *Bacillus anthracis*. Gene 69, 287–300.

[5] Makino, S., Uchida, I., Terakado, N., Sasakawa, C. and Yoshikawa, M. (1989) Molecular characterization and protein analysis of the cap region, which is essential for encapsulation in *Bacillus anthracis*. J. Bacteriol. 171, 722–730.

[6] Reif, T.C., Johns, M., Pillai, S.D. and Carl, M. (1994) Identification of capsule-forming *Bacillus anthracis* spores with the PCR and a novel dual-probe hybridization format. Appl. Environ. Microbiol. 60, 1622–1625.

[7] Hutson, R.A., Duggleby, C.J., Lowe, J.R., Mancher, R.J. and Turnbull, P.C.B. (1993) The development and assessment of DNA and oligonucleotide probes for the specific detection of *Bacillus anthracis*. J. Appl. Bacteriol. 75, 463–472.

[8] Makino, S.-I., Iinuma-Okada, Y., Maruyama, T., Ezaki, T., Sasakawa, C. and Yoshikawa, M. (1993) Direct detection of *Bacillus anthracis* DNA in animals by polymerase chain reaction. J. Clin. Microbiol. 31, 547–551.

[9] Turnbull, P.C.B., Hutson, R.A., Ward, M.J., Jones, M.N., Quinn, C.P., Finnie, N.J., Duggleby, C.J., Kramer, J.M. and Melling, J. (1992) *Bacillus anthracis* but not always anthrax. J. Appl. Bacteriol. 72, 21–28.

[10] Kaneko, T., Nozaki, R. and Aizawa, K. (1978) Deoxyribonucleic acid relatedness between *Bacillus anthracis*, *Bacillus cereus* and *Bacillus thuringiensis*. Microbiol. Immunol. 22, 639–641.

[11] Ash, C. and Collins, M.D. (1992) Comparative analysis of 23S ribosomal RNA gene sequences of *Bacillus anthracis* and emetic *Bacillus cereus* determined by PCR-direct sequencing. FEMS Microbiol. Lett. 94, 75–80.

[12] Bourque, S.N., Valero, J.R., Lavoie, M.C. and Levesque, R.C. (1995) Comparative analysis of the 16S to 23S ribosomal intergenic spacer sequences of *Bacillus thuringiensis* strains and subspecies and closely related species. Appl. Environ. Microbiol. 61, 1623–1626.

[13] Henderson, I., Duggleby, C.J. and Turnbull, P.C.B. (1994) Differentiation of *Bacillus anthracis* from other *Bacillus cereus* group bacteria with the PCR. Int. J. Syst. Bacteriol. 44, 99–105.

[14] Henderson, I., Dongzheng, Y. and Turnbull, P.C.B. (1995) Differentiation of *Bacillus anthracis* and other '*Bacillus cereus* group' bacteria using IS*231*-derived sequences. FEMS Microbiol. Lett. 128, 113–118.

[15] Harrell, L.J., Andersen, G.L. and Wilson, K.H. (1995) Genetic variability of *Bacillus anthracis* and related species. J. Clin. Microbiol. 33, 1847–1850.

[16] Andersen, G.L., Simchock, J.M. and Wilson, K.H. (1996) Identification of a region of genetic variability among *Bacillus anthracis* strains and related species. J. Bacteriol. 178, 377–384.

[17] Patra, G., Sylvestre, P., Ramisse, V., Therasse, J. and Guesdon, J.-L. (1996) Specific oligonucleotide primers for rapid identification of *Bacillus anthracis* strains. In: Proceedings of the International Workshop on Anthrax (Turnbull, P.C.B., Ed.), Salisbury Medical Bulletin – Special Supplement 87, pp. 45–46.

[18] Fellows, P. (1995) A survey of worldwide strains of *Bacillus anthracis*. In: Proceedings of the International Workshop on Anthrax (Turnbull, P.C.B., Ed.), Salisbury Medical Bulletin – Special Supplement 87, pp. 31–33.
[19] Breslauer, K.J., Frank, R., Blocker, H. and Marky, L.A. (1986) Predicting DNA duplex stability from the base sequence. Proc. Natl. Acad. Sci. USA 83, 3746–3750.
[20] Titball, R.W., Turnbull, P.C.B. and Hutson, R.A. (1991) The monitoring and detection of *Bacillus anthracis* in the environment. J. Appl. Bacteriol. Symp. (Suppl.) 70, 9S–18S.

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Molecular Characterization and Protein Analysis of the *cap* Region, Which Is Essential for Encapsulation in *Bacillus anthracis*

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By using genetic complementation tests with various in vitro-constructed mutants with mutations in the *cap* region (which is essential for encapsulation in *Bacillus anthracis*), we identified three cistrons, *capB*, *capC*, and *capA*, in this order of arrangement. Minicell analysis revealed that these cistrons produce proteins of 44, 16, and 46 kilodaltons, respectively. The complete nucleotide sequence of 3,244 base pairs covering the whole *cap* region was determined and revealed the existence of the three open reading frames of *capB* (397 amino acid residues; molecular weight, 44,872), *capC* (149 amino acid residues; molecular weight, 16,522), and *capA* (411 amino acid residues; molecular weight, 46,420) arranged in the order predicted by complementation tests. These three cistrons were all transcribed in the same direction from promoters unique to each cistron. Judging from the predicted amino acid sequence of the three proteins and from their localization and their sensitivity to various physicochemical treatments, they appeared to be membrane-associated enzymes mediating the polymerization of D-glutamic acid via the membrane. Capsular peptides immunologically identical to that of *B. anthracis* were found in *B. subtilis*, *B. megaterium*, and *B. licheniformis*, but no sequence homologous to the *cap* region was found in any of these bacilli other than *B. anthracis*. Using strains of *B. anthracis* with or without insertional inactivation of the *cap* region, we found that the capsule of *B. anthracis* conferred strong resistance to phagocytosis upon the bacterial host.

Bacillus anthracis is the causative agent of anthrax. This is primarily a disease of domestic and wild animals, but in humans exposed to diseased animals the infection manifests as a skin infection or rarely a pulmonary infection or septicemia. Two major virulence factors of *B. anthracis*, a capsule and a tripartite toxin consisting of protective antigen, edema factor, and lethal factor, are known (2, 36, 37, 43). The former is encoded by the 60-megadalton (MDa) plasmid (9, 46), and the latter is encoded by the 110-MDa plasmid (27).

The role of the capsule as a virulence factor has been investigated rather extensively for some bacterial species; these systems include the K1 antigen of *Escherichia coli* (6), the capsule of *Neisseria meningtidis* (21), and the capsule of *Franciscella tulafensis* (30). In these bacteria the capsule confers serum resistance or resistance to phagocytosis. A similar biological role of the capsule is generally assumed in *B. anthracis*, but no reliable evidence has yet been reported. The capsule of *B. anthracis* is unique in that it is composed of a homopolypeptide of D-glutamic acid (11). Other bacilli, such as *B. subtilis* (4), *B. megaterium* (10), *B. licheniformis* (44), *Sporosarcina halophilia* (18), and *Planococcus halophilus* (18), also have a capsule consisting of a homopolypeptide of D- or L-glutamic acid. The immunological and genetic relatedness among these polyglutamate capsules has not been determined. D-Glutamyl transpeptidase in *B. subtilis* (*natto*) (12) and membrane-associated glutamylpolypeptide synthetase in *B. licheniformis* (44) have been reported to play a part in the synthesis of these polyglutamate capsules. However, thus far, nothing is known about the synthetic pathway of capsule formation in *B. anthracis*.

We have made a restriction enzyme cleavage map of pTE702, a 60-MDa plasmid essential for the encapsulation in *B. anthracis* (45), cloned the genetic determinants required for encapsulation (*cap* region) in *E. coli*, and transformed the cloned *cap* region back into *B. anthracis* cured of the 110- and 60-MDa plasmids to examine the expression in the original host bacteria (23, 24). *B. anthracis* and *E. coli* but not *B. subtilis* were found to express an immunologically identical capsular peptide on the bacterial surface upon introduction of the cloned *cap* region.

In the present study the *cap* region was further characterized by cistron analysis, nucleotide sequence determination, minicell product analysis, promoter search, and localization of the products under various experimental conditions. These studies have identified three cistrons, *capB*, *capC*, and *capA*, in this order of arrangement and with a promoter unique to each cistron. Although the capsules of *B. anthracis* and other bacilli were found to be immunologically identical, no genetic homology with the *cap* region was detected in bacilli other than *B. anthracis*.

MATERIALS AND METHODS

Bacterial strains and vector plasmids used. Bacterial strains and representative plasmids are listed in Table 1. pCAP1, pCAP2, and pTPCAP1 were used as parental plasmids containing the intact *cap* region (24).

Construction of plasmids. Deletions 5-5, 5-6, 5-9, 5-3, and 5-10 were constructed by BAL31 treatment of *Bgl*II-digested pCAP2 and *Sal*I linker insertion. Deletions 2-3, 2-22, 2-46, and 2-33 were constructed by BAL31 treatment of *Sal*I-digested pCAP1 and *Bgl*II linker insertion. A *Bgl*II fragment of deletion 2-22 was inserted in the *Bam*HI site of pMYSH6003 to obtain pTPCAP1.

To construct the pCAP and pTPCAP series of plasmid mutants, we used the following three methods: (i) filling in of *Hin*dIII, *Xba*I, *Bam*HI, and *Bst*EII sites with the Klenow fragment; (ii) insertion of a *Bgl*II linker into each of the

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TABLE 1. Bacterial strains and representative plasmids used

Strain or plasmid	Relevant characteristic[a]	Reference or source
E. coli		
MC1061	*hsr lac leu*	7
C600 recA	*recA*	This laboratory
CC118	*phoA*	26
M2124	Minicell-producing cell	39
B. anthracis		
TE702	Spo^- Cap^+ Tox^-	24
TE703	As TE702 but cured of the Cap plasmid	24
TE7021	As TE703 but containing pTE7021	24
Shikan	Virulent strain	45
B. subtilis NIAH801	Cap^+	Product *natto*
B. megaterium NIAH368	Cap^+	Human stool
B. licheniformis NIAH227	Cap^+	Human stool
Plasmids		
pHY300PLK	Ap^r Tc^r pACYC177 derivative	14
pMY6003	Ap^r Tp^r pBR322 derivative	22
pBR322	Ap^r Tp^r	3
pCAP1	*cap* region cloned into pHY300PLK	24
pCAP2	Self-cloning of pCAP1 with *BglII*	24
pTPCAP1	*cap* region cloned into pMY6003	This study

[a] Abbreviations: Spo^-, asporogenous; Cap^+, encapsulated; Cap^-, nonencapsulated; Tox^-, non-toxigenic; Ap^r, ampicillin resistance; Tc^r, tetracycline resistance; Tp^r, trimethoprim resistance.

*Hin*cII, *Pvu*II, and *Aat*I sites; and (iii) deletion of four nucleotides from the *Pst*I site by using the Klenow fragment.

pCAPA and pCAPB were constructed by partial digestion of pCAP2 and pCAP1 with *Pst*I, respectively. pCAPC was constructed by inserting the *Bam*HI-*Bgl*II fragment of deletion 2-33 into the *Bam*HI site of pHY300PLK.

Minicell analysis. Minicells were isolated and labeled with [^{35}S]methionine (Amersham Corp., Amersham, United Kingdom) by the method of Thompson and Achtman (39). For fractionation of the protein products, sodium dodecyl sulfate–12.5% polyacrylamide gels were used.

Nucleotide sequencing. The dideoxynucleotide procedure of Sanger et al. was used for nucleotide sequencing (31). Single-stranded pUC118 DNA was purified and used for sequencing by the Kilo-sequence deletion kit and 7-DEAZA sequencing kit (Takara Shuzo, Co., Kyoto, Japan).

Cell fractionation and trypsin treatment. Labeled minicells were treated with lysozyme (15) at 4°C, and spheroplast and periplasmic preparations were isolated by microcentrifugation at 15,000 × *g* for 30 min at 4°C. The spheroplast preparation was sonically disrupted in the presence of phenylmethylsulfonyl fluoride. Then, membrane and cytoplasmic fractions were isolated by centrifugation at 70,000 rpm for 45 min in a TLA-100.2 rotor (Beckman Instruments, Inc., Fullerton, Calif.). For trypsin digestion experiments for topological mapping (34), spheroplasts were lysed by sonication after freezing and thawing. Intact and lysed spheroplasts were reacted with trypsin in 3 mM EDTA buffer (pH 7.5) at 37°C for 30 min. The reaction was terminated by addition of an excess amount of soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, Mo.), and proteins were precipitated with 5% trichloroacetic acid. Portions were analyzed on sodium dodecyl sulfate–12.5% polyacrylamide gels.

Dot hybridization. Total DNAs from *B. anthracis*, *B. megaterium*, *B. subtilis*, and *B. licheniformis* were prepared by the method of Johnson (16), except that the lysozyme concentration was 10 mg/ml and the treatment was carried out for 90 min at 37°C for *B. anthracis* and 30 min for other strains. To label the probes with [^{35}P]dCTP (Amersham Corp.), we used a random-priming labeling kit (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Total DNAs were transferred to nitrocellulose filters by the method of Maniatis et al. (25). Hybridization was carried out in the presence of 50% formamide at 42°C.

Tn*phoA* insertion experiments. Tn*phoA* (26) was transposed to a suicide plasmid, pCHR81 (32), to construct pCHR71::Tn*phoA*. pCHR71::Tn*phoA* was conjugally introduced into *E. coli* MC1061 containing pCAP1 at 30°C. A Km^r Tp^r transconjugant was spread on an agar plate to yield about 500 separate colonies at 30°C. Each of these isolates was grown at 30°C in L broth containing ampicillin (50 μg/ml) and kanamycin (50 μg/ml), spread on an agar plate containing kanamycin (400 μg/ml), and then grown at 42°C. Cells on the plate were collected, and plasmid DNA was isolated and used to transform *E. coli* CC118. Transformants were selected on an agar plate supplemented with 5-bromo-4-chloro-3-indolyl phosphate (XP; 40 μg/ml) (Sigma) and kanamycin (50 μg/ml) at 37°C. Blue and light-blue colonies were selected and analyzed.

Phagocytosis experiments. Phagocytosis experiments were performed mainly by the method described by Beachey et al. (1). Fresh human blood was heparinized, and erythrocytes were removed by sedimentation in 2% (wt/vol) dextran at 37°C for 40 min. Buffy-coat leukocytes were recovered by centrifugation (400 x *g* for 10 min) and suspended at 56°C for 30 min in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum. Then, 20 μl of bacterial cells was mixed with 200 μl of leukocytes, and the mixture was incubated for 30 min at 37°C with gentle shaking. Smears were examined microscopically.

Other procedures. Media, reagents, isolation of plasmid DNA, and transformation were as described previously (24).

RESULTS

Genetic and molecular identification of the *cap* region on the Cap plasmid. An approximately 6.2-kilobase fragment of the

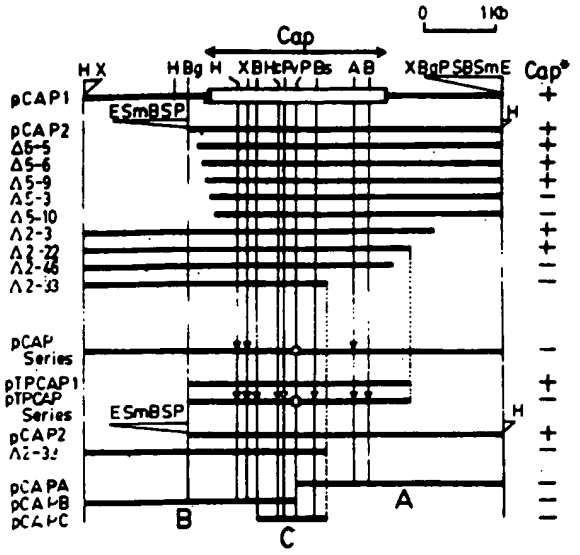

FIG. 1. Physical and genetic map of the *cap* region. In the pCAP and pTPCAP series, pHY300PLK and pMY6003, respectively, were used as vector plasmids. A multicloning site of pHY300PLK is shown at both termini of pCAP1 and pCAP2. Cap* indicates the ability to produce the capsule; positive and negative reactions are judged by reactions with anti-Cap serum, respectively (24). Symbols: ▼, 4-bp insertion; ▽, *Bgl*II linker insertion; □, 4-bp deletion; △, deletion or frameshift mutation; ——, regions present on these plasmids (interruption in each line indicates the deletion); A, B, C, *capA*, *capB*, and *capC*; H, *Hin*dIII; Bg, *Bgl*II; X, *Xba*I; B, *Bam*HI; Hc, *Hin*cII; Pv, *Pvu*II; P, *Pst*I; Bs, *Bst*EII; A, *Aat*I; S, *Sal*I; Sm, *Sma*I; E, *Eco*RI.

cap region of the Cap plasmid, pTE702, which is required for encapsulation in *B. anthracis* TE702, had been cloned in *E. coli* and shown to phenotypically express a capsule substance immunologically identical to that of wild-type *B. anthracis* TE702 (24). To identify and localize the minimum essential region more precisely, various deletion mutants were made by exonucleolytic cleavage with BAL 31 and frameshift mutants were made by in vitro site-specific mutagenesis at the specific restriction sites (Fig. 1). It was identified as an approximately 3.0-kilobase-pair region on pCAP1 (Fig. 1). To analyze the genetic organization of the minimum essential region thus identified, we performed cistron analysis in *E. coli* C600 (*recA*) by complementation between the mutants obtained above. These mutants consist of pCAP series and pTPCAP series mutually compatible with one another. The former were mutants of pCAP1 cloned into pHY300PLK, and the latter were derivatives of pTPCAP1 cloned into pMY6003. The complementation tests scored by the immunological reaction to anti-Cap serum resulted in at least three cistrons, which were designated according to the order of arrangement as *capB*, *capC*, and *capA* (the order A, B, and C is based on the molecular weight of the products in minicells and also on the sizes of the open reading frames) (Table 2). To identify the region coding for the three cistrons, pCAPA, pCAPB, and pCAPC (Fig. 1) were constructed by using the vector pHY300PLK and examined for their ability to restore the Cap$^+$ phenotype when coexisting with one of the mutants of the pTPCAP series. The results agreed with those of the complementation tests and showed that pCAPA, pCAPB, and pCAPC contain *capA*, *capB*, and *capC*, respectively (Table 2). This does not necessarily show that each of the cistrons has its own promoter, because it has been suggested that a promoter activity exists in both directions at the multicloning sites of the vector pHY300PLK.

Identification in minicells of proteins encoded by the Cap region. The products encoded by the *cap* region were analyzed in the *E. coli* minicell system (Fig. 2). Four major proteins, CapA (ca. 46 kDa), CapB (ca. 44 kDa), CapB' (ca. 20 kDa), and CapC (ca. 16 kDa), were produced by the intact *cap* region of pTPCAP1 (Fig. 2A, lane 1). The site-specific mutagenesis of the *cap* region at the *Hin*dIII site abolished the production of CapB (Fig. 2A, lane 2). Mutation at the *Xba*I, *Bam*HI, *Hin*cII, or *Pvu*II site resulted in the loss of CapB and CapB' (Fig. 2A, lanes 3 to 6). Mutation at the *Pst*I or *Bst*EII site inactivated the coding capacity of CapC (Fig. 2A, lanes 7 and 8). Finally, mutation at the *Aat*I site abolished the production of CapA (Fig. 2A, lane 9). Among BAL 31 deletion mutants from the right of pCAP2 with respect to the direction shown in Fig. 1, deletion 2-22 produced the whole intact protein products, whereas deletion 2-46 failed to produce CapA (Fig. 2B, lanes 1 and 2). Similarly, deletion 5-9 was intact with respect to the four protein products, but deletions 5-3 and 5-10 did not produce CapB (Fig. 2B, lanes 3 to 5). pCAPA, pCAPB, and pCAPC were found to encode CapA, CapB plus CapB', and CapC, respectively (Fig. 2C, lanes 3 to 5). All these results are consistent with the conclusion that cistrons *capA*, *capB*, and

TABLE 2. Complementation and rescue tests

pCAP series	pTPCAP series[a]								
	capB					*capC*		*capA*	
	▼*Hin*dIII	▼*Xba*I	▼*Bam*HI	▽*Hin*cII	▽*Pvu*II	□*Pst*I	▽*Bst*EII	▽*Aat*I	▼*Bam*HI
▼*Hin*dIII	−	−	−	−	−	+	+	+	+
▼*Xba*I	−	−	−	−	−	+	+	+	+
□*Pst*I	+	+	+	+	+	−	−	+	+
▽*Aat*I	+	+	+	+	+	+	+	−	−
△2-46	+	NT	NT	NT	NT	+	NT	−	−
△2-33	+	NT	NT	NT	NT	+	NT	−	−
△5-10	−	−	−	−	−	+	NT	+	NT
pCAPA	−	−	−	−	−	−	−	+	+
pCAPB	+	+	+	+	+	−	−	−	−
pCAPC	−	−	−	−	−	+	+	−	−

[a] Symbols: +, Cap$^+$; −, Cap$^-$; NT, not tested. The symbols just before the name of each restriction enzyme represent the nature of in vitro-constructed mutants (Fig. 1).

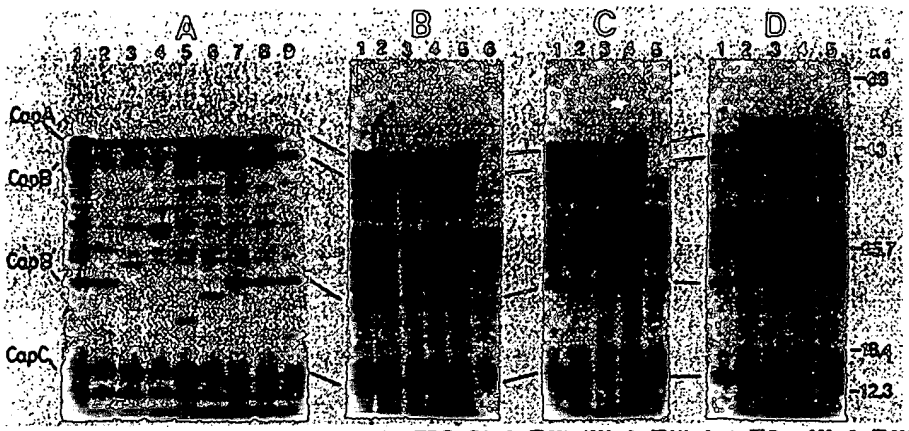

FIG. 2. Protein analysis by minicell methods. (A) Lanes: 1, pTPCAP1; 2, ∇*Hin*dIII; 3, ∇*Xba*I; 4, ∇*Bam*HI; 5, ∇*Hin*cII; 6, ∇*Pvu*II; 7, □*Pst*I; 8, ∇*Bst*EII; 9, ∇*Aat*I. For the significance of each symbol just before the name of each restriction enzyme, see Fig. 1. (B) Lanes: 1, 2-22; 2, 2-46; 3, 5-9; 4, 5-3; 5, 5-10; 6, pHY300PLK. These deletion mutants are derivatives cloned to pHY300PLK. (C) Lanes: 1, pTPCAP1; 2, pCAP1; 3, pCAPB; 4, pCAPA; 5, pCAPC. (D) Lanes: 1, pCAP1; 2, pBRCAP 5-5; 3, pBRCAP 5-6; 4, pBRCAP 5-9; 5, pBRCAP1. These deletion mutants are derivatives cloned to pBRCAP1. Protein products were fractionated on sodium dodecyl sulfate–12.5% polyacrylamide gels. Major proteins, CapA, CapB, CapB', and CapC, are shown on the left side. Positions of marker proteins are shown on the right side. Kd, Kilodaltons.

capC encode CapA, CapB plus CapB', and CapC, respectively.

Complete nucleotide sequence of the *cap* region. The nucleotide sequence of the 3,244 base pairs (bp) covering the whole intact *cap* region and the vicinity of the mutation endpoints of some deletion mutants was determined (Fig. 3). Three open reading frames corresponding to the products CapB (44 kDa), CapC (16 kDa), and CapA (46 kDa) were found in this order and in the same direction. No open reading frame capable of coding for a protein larger than 8 kD was found in the opposite direction. Each coding frame was finally identified on the basis of the following findings. There were three possible translational initiation codons for CapB in frame, the ATG codon at the nucleotide 81 (abbreviated as ATG-81), ATG-282, and GTG-309. Since deletion mutants 5-5, 5-6, and 5-9 were Cap$^+$ but 5-10 lacked the product CapB (Fig. 2B, lanes 3 to 5), ATG-282 was most likely to be the initiation codon for CapB. Thus, CapB contains 397 amino acid residues and has a molecular weight of 44,872. The production of CapB' was abolished by site-specific mutagenesis at any one of the *Xba*I, *Bam*HI, *Hin*cII, or *Pvu*II sites but not at the *Hin*dIII site (Fig. 2A, lanes 2 to 6), indicating that the translational initiation codon for CapB' is GTG-849 in frame with CapB. CapB' consists of 208 amino acid residues and has a molecular weight of 23,349. Since CapB' mutants are always CapB mutants also but the reverse is not the case, the contribution of CapB' to encapsulation cannot easily be decided. However, since the normal production of CapB' always accompanies the normal production of CapB, the physiological function of CapB' may at this stage be considered together with that of CapB. Since CapC disappeared from the minicell products when the *Pst*I site was deleted, the translational initiation codon for CapC appears to be ATG-1490. It consists of 149 amino acid residues and has a molecular weight of 16,522. The translational initiation codon for CapA may be either ATG-1951 or ATG-2134. However, since CapA is appreciably larger than CapB in the minicell analysis, ATG-1951 seems to be more probable. Hence, CapA contains 411 amino acid residues and has a molecular weight of 46,420. For each of the open reading frames described above, one possible ribosome-binding site (Shine-Dalgarno sequence) (35) was found at 5 to 10 bp upstream of the translational initiation codons described above.

Deletion mutant 5-9, with a deletion 274 bp from the *Bgl*II site, leaving only 8 bp up to the translational initiation codon, was found to be Cap$^+$. This is probably due to readthrough from the vector. To identify the promoter unique to the *capB* cistron, we constructed deletion mutants with the vector derived from pBR322 rather than pHY300PLK by exchanging the fragment covering the CapB-coding region. Since a *Sal*I linker had been inserted at the site of deletion endpoints of the pHY300PLK-cloned deletions 5-5, 5-6, 5-9, 5-3, and 5-10, the *Sal*I-*Aat*I fragment of each deletion mutant was exchanged with the *Sal*I-*Aat*I fragment of pBRCAP1 having the intact *cap* region cloned into pBR322 (Fig. 4). Thus, a series of pBRCAP-cloned deletion mutants with the same code number as those of the original pHY300PLK-cloned deletion mutants were obtained. pBRCAP deletion mutant 5-5 was Cap$^+$ like its parental pHY300PLK deletion mutant, 5-5. However, pBRCAP deletion mutants 5-6, 5-9, 5-3, and 5-10 were Cap$^-$, despite the previous finding that the original pHY300PLK deletion mutants 5-6 and 5-9 were Cap$^+$ (Fig. 1). In the minicell analysis, pBRCAP deletion 5-5 produced intact CapA, CapB, and CapC, whereas pBRCAP deletions 5-6 and 5-9 produced intact CapA and CapC but not CapB (Fig. 2D). These observations indicate that the promoter unique to *capB* exists between nucleotides 135 and 239. Since CapA and CapC were phenotypically expressed in minicell analysis of pBRCAP deletions 5-6 and 5-9 in the absence of the promoter unique to *capB*, it seems reasonable to assume that they have their own promoter(s). The putative promoter sequences for each of the three cistrons *capB*, *capC*, and *capA* are shown in Fig. 3. To confirm that these sequences are in fact active as the promoter, the *Bst*EII-*Aat*I fragment, the *Bgl*II-*Hin*dIII fragment, and the *Pvu*II-*Pst*I fragment, containing the putative promoter sequence of *capB*, *capC*, and *capA*, respectively, were each cloned to an expression vector pKK232-8 (5) in the direction of transcription for the chloramphenicol resistance gene. Insertion of either of these sequences increased the MIC of chloramphenicol by about

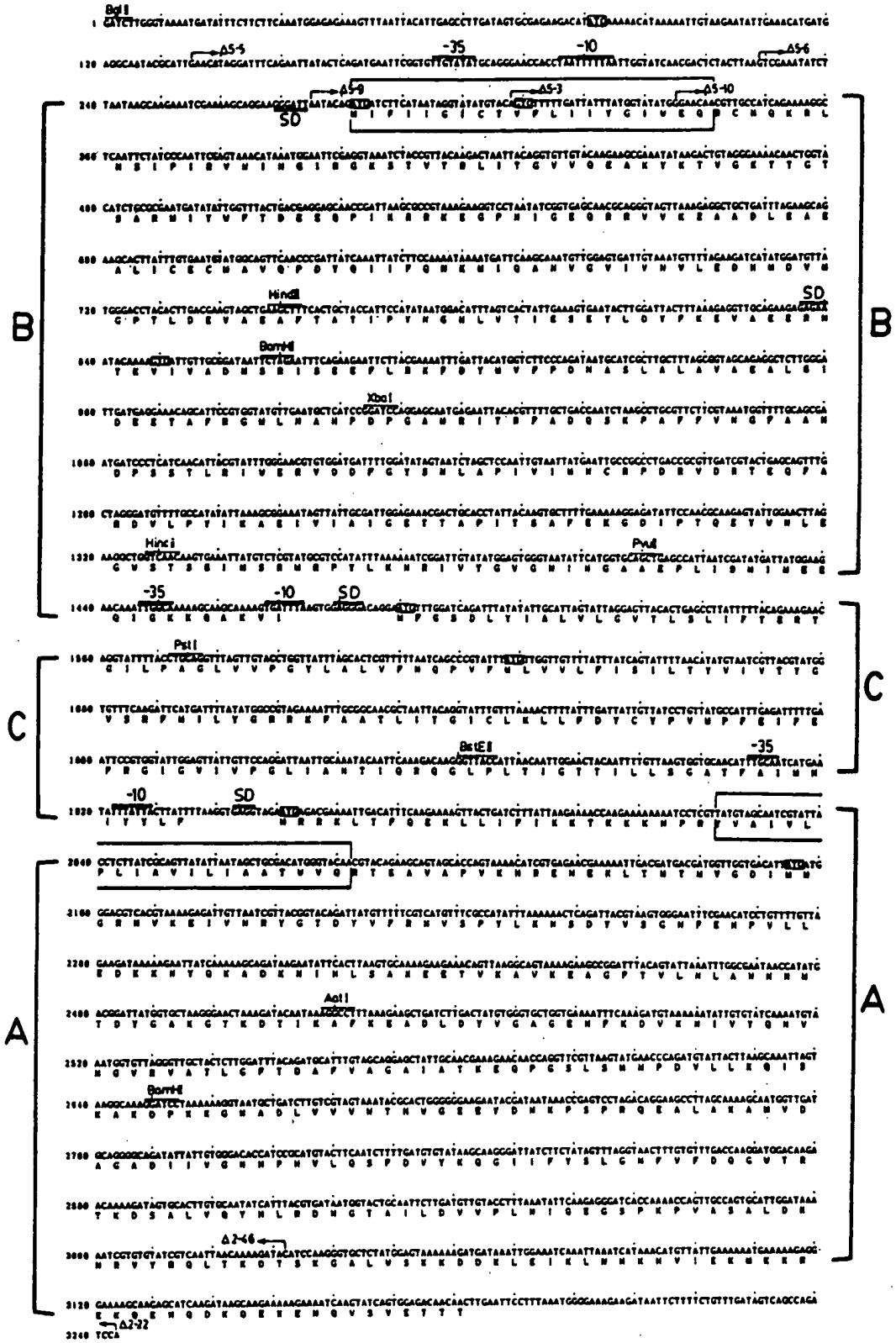

FIG. 3. Nucleotide sequence of the *cap* region. The sequence is numbered starting from the *Bgl*II site of the *cap* region. Possible translational initiation codons are enclosed in small boxes. Possible promoter sequences are shown as −35 and −10, and SD is the possible Shine-Dalgarno sequence (35). Right-directed arrows above the sequence point to the region of DNA remaining undeleted in the denoted deletion mutants (described in the legend to Fig. 1). Hydrophobic amino acid sequences in CapA and CapB are boxed. A, B, and C are the open reading frames of CapA, CapB, and CapC products, respectively.

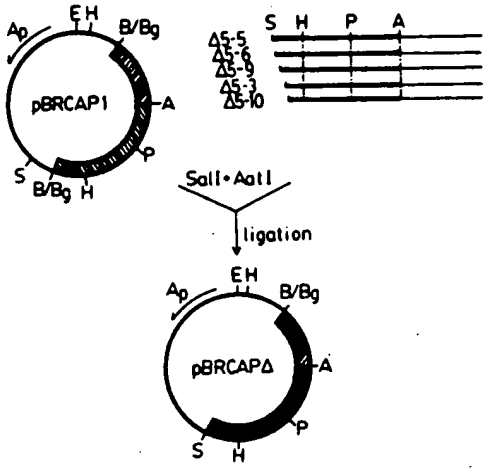

FIG. 4. Construction of pBRCAP1-derived deletions.

4- to 16-fold in *E. coli*. This indicates that the sequences contain a promoter sequence active in *E. coli*. However, the extent of the increase in MIC was not as high as that of the *E. coli*-derived promoter. The introduction of a functional *E. coli* promoter upstream from that of the *B. anthracis cap* region highly potentiated the promoter activity of the latter (data not shown).

Localization of CapA, CapB and CapC proteins in *E. coli*. To determine the location of these proteins, we fractionated the cytoplasmic, membrane, and periplasmic fractions of minicells (Fig. 5A). All the CapA, CapB, and CapC fractions were found in the membrane fraction, as opposed to β-lactamase, which was found in the periplasmic fraction. When the spheroplast preparation was treated with trypsin, all three proteins were unaffected and remained intact, whereas the trypsin treatment following lysis of the spheroplasts by sonication resulted in the loss of the CapA and the CapB proteins (Fig. 5B). These results indicate that all three proteins are membrane-associated proteins.

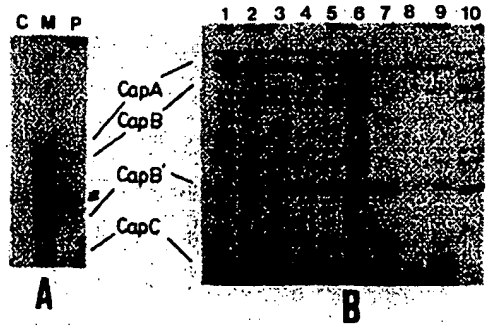

FIG. 5. Fractionation of minicells containing the *cap* region and its trypsin digestion. (A) Fractionation of minicells containing pBRCAP1. C, cytoplasmic fraction; M, membrane fraction; P, periplasmic fraction. An arrow shows the mature β-lactamase protein. (B) Trypsin treatment of spheroplast preparations. Lane 1, intact minicell containing pTPCAP1; Lane 10, periplasmic fraction. The intact (lanes 2 to 5) and the lysed (lanes 6 to 9) spheroplasts derived from minicells containing pTPCAP1 were treated with trypsin at concentrations of 0 (lanes 2 and 6), 5 (lanes 3 and 7), 20 (lanes 4 and 8), or 50 (lanes 5 and 9) μg/ml. Protein products were fractionated on sodium dodecyl sulfate–12.5% polyacrylamide gels.

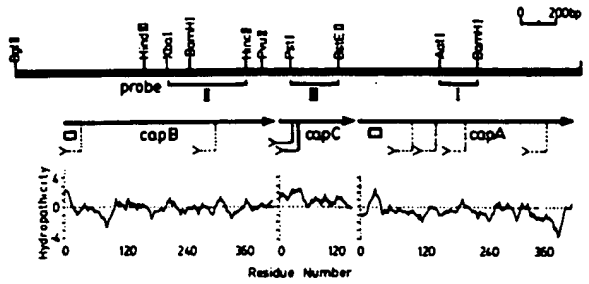

FIG. 6. Protein map of the *cap* region. A hydropathicity plot of each protein was generated by using the program of Kyte and Doolittle (20); window = 20 was used. The value of each position was plotted against the residue number of each protein. Arrows with solid lines at the middle are the open reading frames of the three proteins CapA, CapB, and CapC. Open boxes in CapA and CapB proteins shown near the N terminus are hydrophobic amino acid sequences. Tn*phoA* insertion sites are shown as solid and broken lines below the arrows of open reading frames, and the direction of *phoA* transcription is shown as an arrow; solid and broken lines represent blue and light-blue colonies on XP plates, respectively. Probes I, II, and III were used for dot hybridization experiments (shown in text and in Fig. 7).

Distribution of the sequence homologous to the *cap* region in other bacilli. In addition to *B. anthracis*, *B. subtilis*, *B. megaterium*, and *B. licheniformis* are known to have a capsule consisting of glutamyl polypeptide. To identify the capsular component and its genetic determinant among these bacilli, *B. anthracis* TE702, TE703 (as TE702 but cured of the Cap plasmid), and Shikan (a virulent Cap$^+$ strain), *B. subtilis* NIAH801, *B. megaterium* NIAH368, and *B. licheniformis* NIAH227 were used to prepare the antigen for Ouchterlony precipitation assays against the antibody raised to *B. anthracis* TE702 capsular antigen. Except for the Cap$^-$ TE703 strain, all produced fused precipitation lines (see Fig. 7A), indicating that they produce an immunologically identical polypeptide. On the other hand, dot hybridization was performed with the six strains described above by using three probes, probes I, II and III (Fig. 6 and 7B). Only the Cap$^+$ strains of *B. anthracis*, TE702 and Shikan, were homologous to the probes, indicating that the genetic determinant for the capsular polypeptide in three bacilli other than *B. anthracis* is not homologous to the Cap region.

The *cap* region confers resistance to phagocytosis. Using a pair of Cap$^+$ and Cap$^-$ strains, we examined the biological

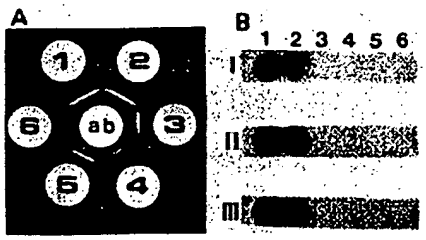

FIG. 7. Distribution of the capsular antigen and the sequence homology to the *cap* region in various bacilli. (A) Ouchterlony assay with anti-Cap serum raised by the capsule antigen from *B. anthracis* TE702 (24). Antigens were isolated by a 20-min autoclaving of the cell suspension grown on NBY agar plates (24) in the presence of 20% CO_2. (B) Dot hybridization with probes I, II, and III (Fig. 6). About 20 μg of total DNA per spot was used. Dots: 1, *B. anthracis* TE702; 2, *B. anthracis* Shikan; 3, *B. subtilis*; 4, *B. megaterium*; 5, *B. licheniformis*; 6, *B. anthracis* TE703.

TABLE 3. Phagocytosis of *B. anthracis* Cap$^+$ and Cap$^-$ strains

Bacterial cells[a]	Treatment[b]	% Phagocytosis[c]
TE702	Untreated	4
TE7021	Untreated	98
TE702	Autoclaved[d]	37
TE7021	Autoclaved[d]	97
TE702	Pronase[e]	55
TE7021	Pronase[e]	93
TE702	Anti-Cap serum[f]	38
TE7021	Anti-Cap serum[f]	96

[a] *B. anthracis* TE702 is Cap$^+$ and contains an intact capsule plasmid pTE702. TE7021 is Cap$^-$ and contains a pTE7021 plasmid with an insertion mutation in the *cap* region.

[b] Because *B. anthracis* cells were linked together, they were slightly sonicated and separated from each other.

[c] Expressed as number of neutrophiles per 100 counted cells containing one or more bacterial cells.

[d] Sonicated cells were autoclaved at 121°C for 30 min and washed in phosphate-buffered saline.

[e] Sonicated cells were treated with pronase (100 μg/ml) at 37°C for 30 min and washed in phosphate-buffered saline.

[f] Sonicated cells were incubated with phagocytic cells in the presence of anti-Cap serum.

role of the capsule. TE702 is a strain of *B. anthracis* that is avirulent as a result of the loss of the toxin plasmid, but it carries the intact capsule plasmid pTE702 and hence is Cap$^+$. TE7021 (24) is a Cap$^-$ derivative of TE702 containing pTE7021 (an insertion mutant in the *cap* region of pTE702). TE7021 but not TE702 was efficiently phagocytosed in the absence of complement and specific antibody (Table 3). Treatments more or less removing the capsular substance from the bacterial surface, such as autoclaving and pronase digestion and the addition of anti-Cap serum to the reaction mixture, slightly but consistently decreased the ability of the Cap$^+$ bacteria to resist phagocytosis.

DISCUSSION

Although a considerable amount of information is available concerning the biosynthesis of bacterial surface structures including capsular polysaccharides (47), lipopolysaccharides (29), peptidoglycan (13), and membrane mannans (33), relatively little is known about the molecular mechanism of the synthesis of the poly-D-glutamyl capsule of *B. anthracis*. We demonstrated in a previous study (24) that the cloned *cap* region was phenotypically expressed in *E. coli* and produced a capsular substance immunologically identical with that of *B. anthracis*. This permitted us to adopt recombinant DNA technology to study the capsule of *B. anthracis* in *E. coli*.

In the present study, to clarify the role of the capsule as a virulence factor of *B. anthracis*, we constructed isogenic Cap$^+$ and Cap$^-$ strains of *B. anthracis* and compared them for the ability to resist phagocytosis. This has clearly demonstrated that encapsulated strains of *B. anthracis* resist phagocytosis.

Furthermore, in the present study, complementation tests, minicell analysis, localization of the products, and nucleotide sequence determination for the *cap* region performed in *E. coli* have identified three cistrons, *capA*, *capB*, and *capC*. Each of these has a respective unique promoter but the same direction of transcription. The hydropathicity of the products, CapA, CapB, and CapC, was plotted (Fig. 6) on the basis of the nucleotide sequences obtained (Fig. 3). CapC was shown to be a hydrophobic protein, strongly suggesting that it is a membrane protein (19). CapA has a hydrophobic segment from Try-25 to somewhere around Gln-44, and the segment is surrounded by Arg. The N-terminal 20 amino acids of CapB are also hydrophobic, and amino acid 21 is Arg (Fig. 3 and 6), suggesting that the N-terminal 20-amino-acid segment is associated with the membrane and the other portion is in the cytoplasmic space. In addition, minicell analysis has revealed that all the CapA, CapB, and CapC fractions are in the membrane fraction (Fig. 5). These results are consistent with the hypothesis that all three proteins are membrane associated; that the hydrophobic segment of CapA and CapB, consisting of 20 amino acids (boxed in Fig. 3), is integrated into the membrane, with the other portion remaining in the cytoplasmic space; and, finally, that CapC is hydrophobic and integrated into the membrane so that its eight trypsin-susceptible sites are protected from digestion by trypsin.

The capsular substance of *B. anthracis*, *B. subtilis*, *B. megaterium*, and *B. licheniformis* was found to be immunologically identical (Fig. 7A). However, the genetic determinant for the synthesis of the capsule substance seems to be distinctive between *B. anthracis* and other bacilli, because no sequence was found in bacilli other than *B. anthracis* that was homologous to any probes derived from the *cap* region (Fig. 7B). Since functionally identical enzymes derived from different species of bacteria may be encoded by genetic determinants completely nonhomologous with one another (17), this observation may not be exceptional.

On the other hand, the lack of homology may reflect the difference in the synthetic pathways. There have been several reports on the enzymatic pathways leading to the biosynthesis of the poly-D-glutamate capsule in bacilli (12, 40, 42, 44). Although the synthetic pathway of capsule formation in *B. anthracis* remains obscure, the capsule of this organism is composed of only a homopolypeptide of D-glutamic acid (11). In contrast, glutamyl polypeptide produced by *B. subtilis* (*natto*) contains both D- and L-glutamic acid (41), and it was reported that γ-glutamyltranspeptidase might participate in the synthesis of capsular substance in *B. subtilis* (12). However, *E. coli* strains (38) defective in γ-glutamyltranspeptidase activity could not be complemented by the *cap* region of *B. anthracis* with respect to this enzymatic defect, but they synthesized the capsule to the same extent as did *E. coli* strains proficient in this enzymatic activity and carrying the *cap* region. Furthermore, in *E. coli* strains defective in this enzyme, the capsular synthesis directed by the *cap* region was not inhibited by the inhibitor 6-diazo-5-oxo-norleucine, which is specific to this enzyme (28) (data not shown). These observations indicate that the enzyme(s) encoded by the *cap* region does not possess γ-glutamyltranspeptidase activity.

In contrast, the existence of membrane-associated polyglutamyl synthetase complex in *B. licheniformis* was reported (8, 44). In experiments with purified membrane fractions of *B. licheniformis*, the reactions of this enzymatic complex were found to proceed sequentially by several apparently separable steps. Thus, L-glutamate is activated in the presence of ATP to form the activated intermediate L-glutamyl AMP. Subsequently, the stereochemical inversion of the L to the D form occurs independently of the activation step. A protein-bound thioester mediates racemization and the subsequent transfer to growing polymeric chains. Several experimental observations have also been reported which support the involvement of an endogeneous acceptor of the N terminus of growing nascent chains onto which sequential addition of activated glutamyl residues occurs (8).

Our preliminary data have revealed that *E. coli* carrying the *cap* region incorporates appreciably higher L-[^{14}C]Glu levels than *E. coli* without the *cap* region does (data not shown). This indicates that L-Glu may be the direct precursor of the capsule encoded by the *cap* region, as is the case in *B. licheniformis*.

Moreover, since the three proteins, CapA, CapB, and CapC, encoded by the *cap* region are all membrane associated (Fig. 5), it is conceivable that the *cap* region codes for a membrane-associated polyglutamyl synthetase complex similar to that in *B. licheniformis*. In *B. anthracis*, the capsular polymer could not be detected in any in vitro-constructed Cap$^-$ mutants (Fig. 1), even after lysis by sonication (data not shown). That is, without all of the three products from the three cistrons, the capsule substance cannot be synthesized at all. This is not compatible with the possibility that the polymerized capsule is synthesized in the cytoplasm and transported to the bacterial surface but suggests that polymerization of glutamate occurs in the membrane. Also, in this respect, the synthetic pathway of the capsular substance encoded by the *cap* region in *B. anthracis* seems to resemble that in *B. licheniformis* (8).

The three proteins in *B. anthracis* were found to be associated with the membrane (Fig. 5). CapC is a hydrophobic protein and seems to exist in membrane-integrated form (Fig. 6). The insertion of Tn*phoA* into the *capC* cistron resulted in a mutant producing a blue colony on an XP plate, which was used for scoring alkaline phosphatase activity, whereas the insertion into *capB* and *capA* resulted in light-blue mutants in the same assay (Fig. 6). These observations indicate that the membrane protein CapC exists neither in the inner membrane nor in the periplasmic space but in the outer membrane in *E. coli*. CapC, but not the two other proteins, was in fact detected in the outer membrane fraction (data not shown). Although these results were obtained with *E. coli*, CapC, among these three proteins encoded by the *cap* region, may exist on the outermost surface in *B. anthracis* also. Thus, if the synthetic pathway in *B. anthracis* highly resembles that in *B. licheniformis*, CapC may be the most suitable candidate for the endogeneous acceptor of glutamyl residues.

ACKNOWLEDGMENT

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LITERATURE CITED

1. Beachey, E. H., and I. Ofek. 1976. Epithelial cell binding of group A streptococci by lipoteichoic acid on fimbriae denuded of M protein. J. Exp. Med. 143:759–771.
2. Beall, F. A., M. J. Taylor, and C. B. Thorne. 1962. Rapid lethal effect in rats of a third component found upon fractionating the toxin of *Bacillus anthracis*. J. Bacteriol. 83:1274–1280.
3. Bolivar, F., R. L. Rodriquez, P. J. Green, M. C. Betlach, H. L. Heyneker, H. W. Boyer, J. H. Crosa, and S. Falkow. 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene 2:95–113.
4. Bovarnick, M. 1942. The formation of extracellular D-glutamic acid polypeptide by *Bacillus subtilis*. J. Biol. Chem. 145: 415–424.
5. Brosius, J. 1984. Plasmid vectors for the selection of promoters. Gene 27:151–160.
6. Cabello, F. C. 1979. Determinants of pathogenicity of *E. coli* K1, p. 155–160. *In* K. N. Timmis and A. Puhler (ed.), Plasmids of medical, environmental and commercial importance. Elsevier/North Holland Publishing Co., Amsterdam.
7. Casadaban, M., and S. N. Cohen. 1980. Analysis of gene control signals by DNA fusion and cloning in *E. coli*. J. Mol. Biol. 138:179–207.
8. Gardner, J. M., and F. A. Troy. 1979. Chemistry and biosynthesis of the poly (γ-D-glutamyl) capsule in *Bacillus licheniformis*. J. Biol. Chem. 254:6262–6269.
9. Green, B. D., L. Battisti, T. M. Koehler, C. B. Thorne, and B. E. Ivins. 1985. Demonstration of a capsule plasmid in *Bacillus anthracis*. Infect. Immun. 49:291–297.
10. Guex-Holzer, S., and J. Tomcsik. 1956. The isolation and chemical nature of capsular and cell-wall haptens in a *Bacillus* species. J. Gen. Microbiol. 14:14–25.
11. Hanby, W. E., and H. N. Rydon. 1946. The capsule substance of *Bacillus anthracis*. Biochem. J. 40:297–309.
12. Hara, T., and S. Ueda. 1982. Regulation of polyglutamate production in *Bacillus subtilis* (*natto*): transformation of high PGA productivity. Agric. Biol. Chem. 46:2275–2281.
13. Higashi, Y., J. L. Strominger, and C. C. Sweeley. 1967. Structure of a lipid intermediate in cell wall peptidoglycan synthesis: a derivative of a C_{55} isoprenoid alcohol. Proc. Natl. Acad. Sci. USA 57:1978–1984.
14. Isiwa, H., and N. Tsuchida. 1984. New shuttle vectors for *Escherichia coli* and *Bacillus subtilis*. 1. Construction and characterization of plasmid pHY460 with twelve unique cloning sites. Gene 32:129–134.
15. Ito, K., T. Sato, and T. Yura. 1977. Synthesis and assembly of the membrane proteins in *E. coli*. Cell 11:551–559.
16. Johnson, J. L. 1981. DNA isolation, p. 452–453. *In* P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Kreig, and G. B. Phillips (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
17. Jones, B. D., and H. L. T. Mobley. 1987. Genetic and biochemical diversity of ureases of *Proteus*, *Providencia*, and *Morganella* species isolated from urinary tract infection. Infect. Immun. 55:2198–2203.
18. Kandler, O., H. Konig, J. Wiegel, and D. Claus. 1983. Occurrence of poly-γ-D-glutamic acid and poly-γ-L-glutamine in the genera *Xanthobacter*, *Flexithrix*, *Sporosarcina* and *Planococcus*. Syst. Appl. Microbiol. 4:34–41.
19. Klein, P., M. Kanehira, and C. DeLisi. 1985. The detection and classification of membrane-spanning proteins. Biochim. Biophys. Acta 815:468–476.
20. Kyte, J., and R. F. Doolittle. 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. 157:105–132.
21. Liu, T. Y., E. C. Gotschlich, E. K. Jonssen, and J. R. Wysocki. 1979. Studies on the meningococcal polysaccharides. I. Composition and chemical properties of the group A polysaccharide. J. Biol. Chem. 246:2849–2857.
22. Makino, S., C. Sasakawa, K. Kamata, T. Kurata, and M. Yoshikawa. 1986. A genetic determinant required for continuous reinfection of adjacent cells on large plasmid in *S. flexneri* 2a. Cell 46:551–555.
23. Makino, S., C. Sasakawa, :. Uchida, N. Terakado, and M. Yoshikawa. 1987. Transformation of cloning vector, pUB110, into *Bacillus anthracis*. FEMS Microbiol. Lett. 44:45–48.
24. Makino, S., C. Sasakawa, I. Uchida, N. Terakado, and M. Yoshikawa. 1988. Cloning and CO_2-dependent expression of the genetic region for encapsulation from *Bacillus anthracis*. Mol. Microbiol. 2:371–376.
25. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. Manoil, C., and J. Beckwith. 1985. Tn*phoA*: a transposon probe for protein export signals. Proc. Natl. Acad. Sci. USA 82: 8129–8133.
27. Mikesell, P., B. E. Ivins, J. D. Ristoph, and T. M. Dreier. 1983. Evidence for plasmid-mediated toxin production in *Bacillus anthracis*. Infect. Immun. 39:371–376.
28. Nakayama, R., H. Kumagai, and T. Tochikura. 1984. Leakage of glutathione from bacterial cells caused by inhibition of γ-glutamyltranspeptidase. Appl. Environ. Microbiol. 47:653–657.
29. Osborn, M. J. 1969. Structure and biosynthesis of the bacterial cell wall. Annu. Rev. Biochem. 38:501–538.
30. Sandstrom, G., S. Lofgren, and A. Tarnvik. 1988. A capsule-

deficient mutant of *Francisella tularensis* LVS exhibits enhanced sensitivity to killing by serum but diminished sensitivity to killing by polymorphonuclear leukocytes. Infect. Immun. 56:1194–1202.
31. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463–5467.
32. **Sasakawa, C., and M. Yoshikawa.** 1987. A series of Tn*5* variants with various drug-resistance markers and suicide vector for transposon mutagenesis. Gene 56:283–288.
33. **Scher, M., and W. J. Lennarz.** 1969. Studies on the biosynthesis of mannan in *Micrococcus lysodeikticus*. I. Characterization of mannan-^{14}C formed enzymatically from mannosyl-1-phosphoryl-undecaprenol. J. Biol. Chem. 244:2777–2789.
34. **Shiba, K., K. Ito, T. Yura, and D. P. Cerretti.** 1984. A defined mutation in the protein export gene within the *spc* ribosomal protein operon of *Escherichia coli*: isolation and characterization of a new temperature-sensitive *secY* mutant. EMBO J. 3:631–635.
35. **Shine, J., and L. Dalgarno.** 1974. The 3'-terminal sequence of *Escherichia coli* 16S ribosomal RNA: complementarity to nonsense triplets and ribosome binding sites. Proc. Natl. Acad. Sci. USA 71:1342–1346.
36. **Stanley, J. L., K. Sargeant, and H. Smith.** 1960. Purification of anthrax toxin factors I and II of the anthrax toxin produced *in vitro*. J. Gen. Microbiol. 22:206–218.
37. **Sterne, M.** 1937. Variation in *Bacillus anthracis*. Onderstepoort J. Vet. Sci. Anim. Ind. 8:279–349.
38. **Suzuki, H., H. Kumagai, and T. Tochikura.** 1987. Isolation, genetic mapping, and characterization of *Escherichia coli* K-12 mutants lacking γ-glutamyltranspeptidase. J. Bacteriol. 169: 3926–3931.
39. **Thompson, R., and M. Achtman.** 1978. The control region of the F sex factor DNA transfer cistrons: restriction mapping and DNA cloning. Mol. Gen. Genet. 165:295–304.
40. **Thorne, C. B., C. G. Gomez, and R. D. Housewright.** 1955. Transamination of D-amino acids by *Bacillus subtilis*. J. Bacteriol. 69:357–362.
41. **Thorne, C. B., and C. G. Leonard.** 1958. Isolation of D- and L-glutamyl polypeptides from culture filtrates of *Bacillus subtilis*. J. Biol. Chem. 233:1109–1112.
42. **Thorne, C. B., and D. M. Molnar.** 1955. D-Amino acid transamination in *Bacillus anthracis*. J. Bacteriol. 70:420–426.
43. **Thorne, C. B., D. M. Molnar, and R. E. Strange.** 1960. Production of toxin in vitro by *Bacillus anthracis* and its separation into two components. J. Bacteriol. 79:450–455.
44. **Troy, F. A.** 1973. Chemistry and biosynthesis of the poly(γ-D-glutamyl) capsule in *Bacillus licheniformis*. J. Biol. Chem. 248:305–315.
45. **Uchida, I., K. Hashimoto, S. Makino, C. Sasakawa, M. Yoshikawa, and N. Terakado.** 1987. Restriction map of a capsule plasmid of *Bacillus anthracis*. Plasmid 18:178–181.
46. **Uchida, I., T. Sekizaki, K. Hashimoto, and N. Terakado.** 1985. Association of the encapsulation of *Bacillus anthracis* with a 60 megadalton plasmid. J. Gen. Microbiol. 131:363–367.
47. **Vijay, I. K., and F. A. Troy.** 1975. Properties of membrane-associated sialyltransferase of *Escherichia coli*. J. Biol. Chem. 250:164–170.

Research Report

Design Strategies and Performance of Custom DNA Sequencing Primers

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ABSTRACT

This study surveyed strategies of sequencing primer selection and evaluated primer performance in automated DNA sequencing. We asked participants to relate their preferred primer design strategies to identify primer characteristics that are considered most important in sequencing primer design. The participants preferred primers of 18–24 nucleotides (nt), 39%–58% G+C, a melting temperature (T_m) of 53°–65°C with a 1–2 nt 3′ GC clamp, hairpin stems of less than 2–3 bp, homopolymeric runs of less than 4–5 nt, primer dimers of less than 3–4 bp and secondary priming sites of less than 3–4 bp. We provided a 300-bp test sequence and asked participants to submit sequences of 1–3 optimal sequencing primers. Submitted primers ranged from 17–24 nt and largely conformed to the preferred parameters. Submitted primers were distributed across the test sequence, although some sites were disfavored. Surprisingly, approximately 45% of the primers were selected "manually", more than by any software package. Each of 69 submitted and 95 control primers, distributed at 3-bp intervals across the test sequence, were synthesized, purified and tested using a Model 377 PRISM™ DNA Sequencer with dichlororhodamine dye terminator reagents (dRhodamine dye terminators). Approximately half of the control primers were also tested using rhodamine dye terminator reagents ("old" rhodamine dye terminators). The results indicated that primer physico-chemical characteristics thought to have a strong impact on sequencing performance had surprisingly little effect. Thus, primers with high or low percent G+C or T_m, strong secondary priming scores or long 3′ homopolymeric stretches yielded excellent sequences with the dRhodamine dye terminator reagents, although these characteristics had a stronger effect when the old rhodamine reagents were used. The old rhodamine reagents gave sequences with a similar average read length, but the number of errors and ambiguities or "N's" was consistently higher. Moreover, the effects of the primer physico-chemical characteristics were also more evident with the old rhodamine dyes. We conclude that under optimal sequencing conditions with highly pure template and primer, many of the commonly applied primer design parameters are dispensable, particularly when using one of the new generation of sequencing reagents such as the dichlororhodamine dye terminators.

INTRODUCTION

The Nucleic Acids Research Committee (NARC) of the Association of Biomolecular Resource Facilities (ABRF) evaluates the procedures and performance of research facilities that perform nucleic acid synthesis and sequencing in academic, industrial and other institutions (5,6). These studies provide a measure of the industry state-of-the-art, as well as the level of performance, that can be expected from such facilities. Previous projects have examined general operations of DNA core laboratories (15), the accuracy of automated DNA sequencing in these facilities (12), and the performance of unpurified synthetic oligonucleotides as primers for automated DNA sequence analysis (14). The DNA Sequencing Research Committee of the ABRF has performed studies evaluating the ability of DNA core facilities to sequence difficult templates (2) and the performance of various sequencing strategies and chemistries (1). Herein, we have studied primer selection strategies used by DNA sequencing facilities and empirically examined the quality of the sequence data provided by primers selected by these facilities.

Despite a long-standing conviction within the DNA sequencing community that careful primer design is essential to ensure high quality data (c.f., 11,14,17,18), there is a paucity of empirical studies supporting this view. A variety of reasonable "rules of thumb" (13) suggesting optimal ranges for primer length, percent G+C, melting

Table 1. Predicted Optimal and Observed Physico-Chemical Characteristics of Primers for Automated DNA Sequencing or PCR

	Sequencing Primers[a]		PCR Primers[b]		Submitted Primers[c]	
	Optimum[d]	Range[e]	Optimum[d]	Range[e]	Mean[f]	Range[g]
Length	18–24 nt	15–40 nt	18–29 nt	15–40 nt	19.7 nt	16–24 nt
T_m[h]	52°–66°C	40°–95°C	50°–69°C	40°–95°C	66°C	48°–91°C
Percent G+C of primer	40%–61%	30%–70%	40%–61%	30%–70%	53%	29%–82%
3′ GC clamp[i]	1–2 nt	0–5 nt	1–2 nt	0–2 nt	1.1 nt	0–4 nt
Primer dimer[j]	3–4 bp	0–7 bp	3–4 bp	0–7 bp	-5.4	-1.6–16.5
Hairpin stems[k]	2–3 bp	0–6 bp	2–3 bp	0–6 bp	1.1 bp	0–5 bp
Homopolymer runs[l]	4–5 nt	3–10 nt	4–5 nt	3–10 nt	3.0 nt	2–5 nt
Secondary priming[m]	3–4	0–10 bp	3–4 bp	0–10 bp	49[n]	0–266[n]

[a]Parameter values preferred by participants for primers to be used in sequencing.
[b]Parameter values preferred for primers to be used in PCR experiments.
[c]Parameter values of the primers submitted by participants.
[d]Optimum values for each parameter as selected by the participants.
[e]The range of acceptable values selected by the participants.
[f]The mean values for each parameter calculated from the submitted primers.
[g]The range of values for each parameter calculated from the submitted primers.
[h]Values submitted by participants for primers to be used for sequencing or PCR and nearest neighbor T_m of primers submitted by participants.
[i]The 3′ clamp refers to the number of G or C bases at the 3′ terminus of the primer.
[j]Largest number of contiguous complementary bases permitting dimerization of primer or the stability of strongest potential primer dimer (kcal) as measured by Oligo 5.0.
[k]Largest number of bases capable of forming a hairpin stem in the primer.
[l]Number of bases in longest homolopolymeric run in the primer.
[m]Number of bases permitted in most stable secondary priming site.
[n]The priming efficiency as calculated by Oligo 5.0 for test sequence only (submitted primers).

temperature (T_m) and composition have led to the development of primer design software packages that identify primers conforming to these criteria (4,16,18). However, the success of these strategies in selection of high-quality sequencing primers, to our knowledge, has not been extensively examined. In many sequencing projects, primer design and synthesis represent the most significant costs and consume the bulk of effort and time. Therefore, it is extremely important that these primers yield high-quality sequence data.

To examine this issue, an e-mail survey containing general questions about laboratory functions and specific questions concerning important characteristics for primer selection was distributed. A 300-bp test sequence was provided for which participants were asked to design sequencing primers. The submitted primers and a set of control primers spanning the 300-bp test sequence at 3-bp intervals were synthesized and used to sequence the test template on a Model 377 PRISM™ Automated DNA Sequencer (PE Biosystems, Foster City, CA, USA). Due to the results of this study, current primer design rules of thumb may be streamlined to facilitate more efficient primer selection for sequencing projects.

MATERIALS AND METHODS

The Survey

In December of 1996 and January of 1997, members of the ABRF were requested via e-mail distributions, the ABRF Electronic Bulletin Board and a standard mailing, to participate in a study of DNA sequencing primer design. The distribution provided a 300-bp "test sequence" (Figure 1) that was selected and previously shown to contain no segments that affect sequence ladder extension (data not shown). Participants were asked to use the prevailing technology in their laboratories to design and submit the sequences of up to three sequencing primers in the forward direction. Participants were requested to select the best primers irrespective of their positions on the test sequence. All responses were anonymously screened and coded by Virginia Commonwealth University (VCU) Health Sciences Computer Center personnel. Identifying data were removed from each document before being forwarded to the Committee for analysis. Several respondees submitted uninterpretable data. Where possible, these respondees were identi-

fied by Computer Center personnel, and new information was requested. Every effort was made to maintain the integrity of the data and the anonymity of the participants.

Control and Submitted Primers

A panel of 95 primers were synthesized as controls. These 18 nucleotide (nt) primers spanned the 300-bp test sequence with their 5′ termini located at 3-bp intervals (i.e., the first began at bp 1 of the test sequence, the second began at bp 4, etc.). All primers were analyzed with the Oligo™ Program 5.0 (NBI/Genovus, Plymouth, MN, USA) (16) and the number of bases, percent G+C, T_m (nearest neighbor method described in Reference 9), internal structure, secondary priming sites, etc., were recorded.

The 69 submitted and 95 control primers were synthesized on a Model 3948 DNA Synthesis and Purification System (PE Biosystems) at three different sites. Synthesis was performed at the 40 nmol scale using PE Biosystems reagents and the standard synthesis, cleavage and purification cycles. An aliquot of each sample was analyzed by polyacrylamide gel electrophoresis (PAGE) and by matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF/MS). PAGE was performed using precast minigels (Novex, San Diego, CA, USA) following the protocol specified by the manufacturer. Mass analysis was performed on a 200-pmol sample mixed with 10 mg/mL 3-hydroxypicolinic acid matrix (in 50% acetonitrile, 0.1% trifluoracetic acid) on a Voyager RP MALDI-TOF/MS instrument (PerSeptive Biosystems, Framingham, MA, USA). Criteria for passing quality control were: (*i*) a single major band on the gel; (*ii*) appropriate mobility on the gel compared to molecular weight standards; (*iii*) a synthesis yield >0.5 ODU (absorption of sample in 1 mL at 260 nm with 1 cm pathlength set up on the 3948 Synthesizer); and (*iv*) a single peak of the expected mass by MALDI-TOF/MS. Any oligonucleotides that failed to meet these criteria were resynthesized.

Test Template and Preparation

The plasmid template was preselected to contain a test sequence lacking obstacles to sequence extension (data not shown) and purified by double banding in CsCl-ethidium bromide isopycnic density gradients (10).

DNA Sequence Analysis

Each of the purified and quality-tested oligonucleotides were used as a primer for DNA sequence analysis using dRhodamine Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® FS reagents (PE Biosystems) under standard reaction conditions as described by the manufacturer at 20 µL total vol containing 300 ng of template and 5 pmol of primer. Sequencing reactions were run for 25 cycles of 10 s at 96°C, 5 s at 50°C and 4 min at 60°C in a GeneAmp® PCR System 9700 DNA Thermal Cycler (PE Biosystems). Reactions were analyzed on Model 377 PRISM Automated DNA Sequencers using 5% Long Ranger™ (FMC BioProducts, Rockland, ME, USA) gels with Tris-Borate-EDTA buffer (TBE; pH 8.3). Approximately 30% of the control primers were rerun using the same dRhodamine dye terminator/Taq FS kit to ensure reproducibility, and approximately 50% of the control primers were rerun using the old rhodamine dye terminator/Taq FS kit (PE Biosystems). The dRhodamine or dichlororhodamine reagents are modifications of the original old rhodamine dyes that have narrower emission spectra, less spectral overlap and more even peak heights than the earlier dyes (19). Each 5% polyacrylamide sequencing gel was electrophoresed for approximately 8 h, and the data was directly exported for analysis using Sequencher™ (Gene Codes, Ann Arbor, MI, USA). Using this software, the raw data was trimmed to remove 5′ and 3′ ambiguous sequences, so that the first and last 25 nt contained no ambiguities. Each result was aligned with the known sequence, and we recorded: (*i*) the number of nt between the 3′ end of the primer and the beginning of the trimmed data; (*ii*) the number of nt in each sequence read; and (*iii*) the number of errors, including miscalls, insertions, deletions and ambiguities in each 100-bp window through the end of the sequence read.

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  1   ATCCACACGG CGCTTTGACT CCCCTTCTGA AAAAAAAGAA CACGTTGATT
 51   CCCCTTTTGA TTTCCCACGA ATGCGAAGCA CAATTCACGC ACAGCCAACG
101   GCAGGGAGAG ACGATAAGGT AAAATACACA CAAGTGGTGG AATCACAAAA
151   GGACACGCAC AGCACGCAAA TAAACATTGC ACACGGCATG ACATTTTGCG
201   ACCCACACAC AATGTGTACA CTACAATTAT ATGAAAATCC CTCCCCCATT
251   CCGCGGGTGC GCCGCAAAGG GCCAAAAACC CAAATGATCC ACTTTATTAT
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Figure 1. Test sequence. Shown is the 300-bp sequence distributed to participants for design of forward sequencing primers. Participants were asked to design primers in the forward direction.

RESULTS

Participant Profiles

Thirty-nine laboratories participated in the study, submitting 69 primer sequences. Of the participants, approximately 77% provide synthesis services and approximately 85% provide sequencing services, but only approximately 50% provide primer design or walking services, and less than one third offer template preparation. Laboratories performing these services averaged annually approximately 3700 syntheses, 7000 sequence runs, 425 primer designs, 424 template purifications and less than 200 primer walking projects. Charges for services averaged $1.17/nt for 40–50 nmol and $2.19/nt for 150–200 nmol syntheses of a 25-nt unpurified primer and $24/reaction for DNA sequence analysis ($9 for gel only). The average price for a primer walking sequencing project of a 2-kbp insert in a plasmid vector was approximately $500 or $0.25/bp. Charges for "external users", where available, averaged approximately 17%–50% higher than charges for institutional users. DNA synthesis and sequencing instrumentation was dominated by PE Biosystems; i.e., 77% of synthesizers and 92% of sequencers, but Beckman

Instruments, PerSeptive Biosystems and LI-COR were also represented. Only approximately 15% of the participants maintained robotic systems for template preparation or sequencing. Finally, when asked if commercial suppliers had an impact on services provided by the facilities, almost 80% of the laboratories responded affirmatively. Approximately 38% had dropped

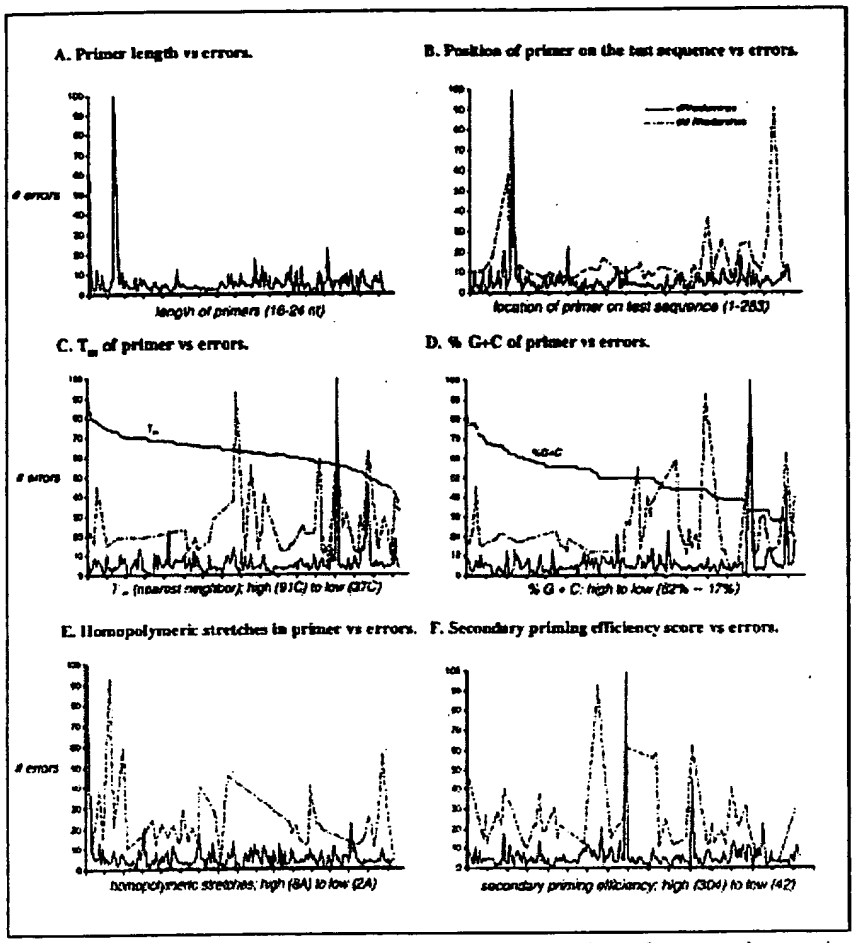

Figure 2. Primer characteristics vs. sequencing performance. The performance of sequencing primers, sorted according to various physico-chemical characteristics, was examined. The number of errors over the full-length read of each primer, determined as described in the Materials and Methods, was plotted against the results of each primer sorted according to the relevant parameter. (A) Primer length vs. errors. The primers were sorted according to their lengths in ascending order, and secondarily, according to their positions on the test sequence. The number of errors over the full-length read is plotted. Note that since all of the primers tested with the old rhodamine reagents were controls, and therefore 18-nt long, the data for old rhodamines are not included in this analysis. The X-axis distributes the primers from 16–24 nt in length; the y-axis indicates the number of errors over the full-length read of that primer. (B) Position of the primer on the test sequence. The primers were sorted according to the position of their 5′ nt on the test sequence (bp 1–283 on x-axis) and the number of errors in their full-length reads were plotted (y-axis). (C) T_m of the primer. The primers were sorted in descending order by their T_m's (91°–37°C on x-axis) and the number of errors in their full-length reads were plotted (y-axis). (D) The percent G+C of the primer. The primers were sorted in descending order (82%–17% on x-axis) by their percent G+C, and the number of errors in their full-length reads were plotted (y-axis). (E) Homopolymeric stretches in the primer. The primers were sorted according to first the number of nt (8–2 on x-axis) in the longest homopolymeric stretch, and secondarily according to the number of nt in the second longest homopolymeric stretch. The number of errors over the full-length read were plotted (y-axis). (F) Secondary priming efficiency of the primers. Each primer was examined for secondary priming sites in the vector and across the test sequence using Oligo 5.0. The priming efficiency score assigned by Oligo 5.0 was determined. The primers were sorted first according to the score of the strongest secondary priming site in the vector (304– 42 on x-axis) and secondarily according to the strongest secondary priming score in the test sequence. The number of errors across the full-length reads were plotted (y-axis).

Table 2. Average Primer Performances of Submitted and Control Primers Using dRhodamine or Old Rhodamine Dye Terminator Sequencing Kits[a]

	Submitted Primers dRhodamines[b]	Control Primers dRhodamines[b]	Control Repetition dRhodamines[b]	Control Primers old kit dyes[b]
5′ nt trimmed[c]	3.3 (2, 1–14)	4.6 (4, 1–19)	4 (3, 0–16)	4.5 (4, 0–16)
read length in nt[d]	760 (770, 674–802)	772 (778, 683–799)	798 (803, 733–867)	752 (764, 654–803)
ambiguities (N's)[e]	2.2 (1, 0–16)	1.6 (1, 0–32)	4.4 (4, 1–17)	8.7 (7, 0–58)
errors per first 700 nt[f]	2.9 (2, 0–14)	2.9 (2, 0–46)	5.4 (5, 3–16)	16.5 (12, 3–92)

[a]Old rhodamine kits refer to the original ABI PRISM Dye Terminator Cycle Sequencing Kits, dRhodamine kits refer to the new ABI PRISM dRhodamine Terminator Cycle Sequencing Ready Reaction Kits containing the dichlororhodamine dye terminators.
[b]The data is shown as the average with the (median, range) in parentheses.
[c]The number of nt between the 3′ end of the primer and the beginning of the trimmed sequence.
[d]The length of read in nt after trimming using Sequencher (see Materials and Methods).
[e]The number of ambiguous calls (N's) in the full-length sequence read.
[f]The number of errors in the first 700 nt of the sequence.

prices and approximately 25% indicated a decreased number of synthesis requests in response to commercial suppliers. About 23% of the laboratories purchased or planned to purchase large-scale synthesis instruments to match the new competition. However, only approximately 5% had discontinued synthesis services.

Software or Manual Selection of Primers

Thirty-one laboratories described their practices for the design of sequencing and PCR primers. We were surprised that, in spite of the availability of excellent commercial primer design software programs, over 50% (17/31) of the reporting laboratories preferred to select primers manually. Of the 25 laboratories reported that selection and design of PCR primers, again over 50% (14/25) tended not to use software. Of those laboratories that rely on software for primer selection, over 60% rely on various versions of the Oligo software for sequence primer design, and over 70% use the Oligo software for PCR primer design.

Idealized Primer Physico-Chemical Characteristics

Participants were asked to provide their preferences for the physico-chemical characteristics of the ideal sequencing and PCR primers. This analysis provided few surprises (Table 1), in that the idealized primer characteristics of most respondents closely parallel those of some common rules of thumb for primer design. Thus, sequencing primers should be: 18–24 nt in length, T_m = 52°–66°C, 40%–60% G+C, with 1–2 bp 3′ GC clamps (G or C nt at 3′ terminus of the primer) and very little internal structure, homopolymeric sequence or potential for secondary priming. A similar set of characteristics was reported for PCR primers, although the optimal acceptable length, T_m and percent G+C were slightly greater than for sequencing primers. However, it was unexpected that some participants accept primers of up to 40 bases, with T_m's as high as 95°C for both sequencing and PCR primers. When asked to rate the relative importance of each characteristic, participants rated the existence of secondary priming sites and percent G+C as most important, with T_m, presence of a 3′ GC clamp, the potential to form primer dimers, primer length, the presence of internal structure or the presence of homopolymeric runs, in order of decreasing importance for sequencing primer design. Relative importance of these characteristics was similar for PCR primers, except that the potential for formation of primer dimers was rated almost as important as the existence of potential secondary priming sites.

Characteristics of the Submitted Primer Sequences

The 39 participating laboratories submitted 69 primer sequences ranging in length from 16–24 nt with an average of approximately 20 nt. The primers were distributed fairly evenly across the 300-bp test sequence, although some segments were obviously purposefully excluded; e.g., any primer beginning between bp 21 and 30 of the test sequence would bear an 8-nt poly(A) segment (not shown). Not surprisingly, most of the submitted primers conformed closely to the idealized physico-chemical characteristics for sequencing primers (Table 1). Although some deviated from the idealized characteristics, all conformed to the more broad ranges defined by participants. Thus, the participants selected primers according to their idealized criteria, even though almost half of the submitted primers were selected manually without the aid of computer software. Interestingly, some of the submitted primers had very high or low T_m (91° or 48°C) or percent G+C (82% or 29%), indicating that the participants do in fact permit a significant variation in these characteristics. Moreover, some primers exhibited relatively high

secondary priming efficiency numbers (a score calculated by Oligo 5.0, whereby a priming efficiency of 160 can prime in sequencing reactions) even within the test sequence (Table 1).

Sequencing Performance of the Primers

The results of the sequence analysis as described in the Materials and Methods were unexpected (Table 2); i.e., we expected a wide range in the performance of the submitted and control primers. Instead, all of the submitted primers functioned extremely well, the poorest performer still yielding <2% errors over a >700-nt sequence. The average number of errors and ambiguities over the 700-nt window was 2.9, and the average read length was 760 nt. Similarly, almost all of the control primers functioned very well, with an average of 2.9 errors or ambiguities over the 700-nt window and an average read length of 772 bases. Only one primer (No. 8) failed to give any reasonable sequence, and even this oligonucleotide performed adequately on the repeat; i.e., 13 errors and ambiguities over a 700-nt read. The average read lengths of the sequences from the submitted and control primers were 760 and 772, with ranges of 674–802 and 683–799 bases, respectively. The average number of nucleotides trimmed from the 5′ ends of these sequences was 3.3 and 4.6 nt, respectively. Many sequences began on the first nucleotide after the primers, but up to 14 or 19 nt were trimmed from the 5′ ends of the submitted and control primers. As expected from previous studies (1,2,12, 14,18), errors and ambiguities were clustered in the first and last 100-bp windows of the sequences (data not shown). Approximately 30% of the control primers were used in repeat reactions with very similar results (Table 2, and data not shown).

The control primers used in sequence reactions using the old rhodamine dye terminator reagents gave slightly poorer overall results. The read lengths of these reactions did not differ greatly from the read lengths of the dRhodamine reactions (average 752 nt vs. 772 bases, Table 2). However, there were more N's (average 8.7 vs. 1.6) and errors and ambiguities (average 16.5 vs. 2.9) in the data generated with old rhodamine dye terminators than in the dRhodamine-generated data (Table 2).

Poor Primers Yield Good Sequence Results

The uniform high-quality sequence data from the control primers using the dRhodamine reagents was not expected. Only primer No. 8 failed catastrophically (i.e., gave no valid sequence data), and primer No. 9 yielded >5% errors over a 700-nt window. However, both of these primers provided better data when used in a repeat sequencing reaction (Table 3). Moreover, there was no obvious relationship between the number of errors in a sequence and the length, position, T_m, percent G+C, secondary priming potential, length of homopolymeric stretches (Figure 2, panels A–F) or the potential for forming primer dimers (data not shown) of the primers. On the contrary, many primers exhibiting suboptimal physico-chemical characteristics yielded high-quality sequence. For example, although the one primer, No. 8, that failed castastrophically has an 8-nt poly(dA) stretch near its 3′ terminus, other primers, e.g., Nos. 7 and 10, contain a similar sequence, but still generated very respectable data (Table 3). Primer No. 9, which also contains the same poly(dA) stretch, also yielded relatively poor data (46 errors in 700 bases), although the error rate was <3% over the first 500-nt window (data not shown). Examination of the sequences of these primers suggests that homopolymeric stretches of dA's may have a negative impact on sequence quality, but that these stretches must be quite long, and near to the 3′ terminus to exert their effects. Other primers with extensive homolopolymeric stretches yielded good results (Table 3). Finally, when the length of the longest homopolymeric stretch in each of the primers was plotted against the number of errors in the sequence reaction (Figure 2E), no general trend was observed when reactions were performed with dRhodamine reagents. Together, these observations suggest that under optimal conditions using dRhodamine reagents, long homopolymeric stretches do not necessarily negatively

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Table 3. Primers with Physico-Chemical Characteristics that Were Not Expected to Provide Good Sequencing Results

Primer#	Sequence			Errors[1]					
				dRhodamines				"old" Rhodamines	
				per 700 nt	total read	per 700 nt	total read	per 700 nt	total read
Homopolymeric stretches:		*length[2]:*							
#6	TGACTCCCCTTCTGAAAA	4A/4C		5	6/754	4	9/803	--	38/685
#7	CTCCCCTTCTGAAAAAAA	7A/4C		4	8/755	6	16/830	10	13/748
#8	CCCTTCTGAAAAAAAAGA	8A/3C		FAILED		13	17/819	--	62/558
#9	TTCTGAAAAAAAAGAACA	8A/2A		--	46/683	7	19/842	57	63/714
#10	TGAAAAAAAAGAACACGT	8A/2A		3	9/786	3	11/790	11	12/721
#76	ATTATATGAAAATCCCTC	4A/3C		2	7/797	nd	nd	nd	nd
#77	ATATGAAAATCCCTCCCC	4C/4A		0	3/772	nd	nd	nd	nd
Melting temperature - %G+C:		*Tm:*	*%G+C*						
#10Ab	CCGCGGGTGCGCCGCAAAGGGC	91°C	82	3	6/773	nd	nd	nd	nd
#84	TCCGCGGGTGCGCCGCAA	83°C	78	1	2/724	6	17/766	10	22/766
#80	TCCCTCCCCCATTCCGCG	75°C	72	5	11/771	5	31/867	15	15/703
#39	TAAGGTAAAATACACACA	44°C	28	2	4/768	7	9/735	12	15/738
#72	GTGTACACTACAATTATA	37°C	28	3	3/763	nd	nd	16	32/780
Secondary priming sites:		*score[3]:*							
#82	CCCCATTCCCCCGGGTGCG || ||||| ||||||| TGGTAAAGGC-CCCACGC	304/518		5	9/767	8	19/823	14	16/709
#86	GGTGCCCCCCAAAGGGCC | |||| ||||||| TCGGTCGGC-CTTCCCGG	274/517		1	8/776	8	14/803	23	46/784
#49b	CGCTTTGACTCCCCTTCTGA | | |||| |||||| ||| GTGCAACTAAGGGGAAAACT	246/451		6	11/784	nd	nd	nd	nd
#10Ab	CCGCGGGTGCGCCGCAAAGGGC | | |||| |||||| TTTGGTCGGTCGGC-CTTCCCG	190/643		3	6/773	nd	nd	nd	nd

[1]Errors and ambiguities per 700 nt read, or per total read length. For the dRhodamines, both the initial results and the results of the repeat analysis are shown.

[2]Length of the longest and second longest homopolymeric stretches in the primer.

[3]X/Y where X is the priming efficiency number generated by Oligo version 5.0, for potential secondary priming in the vector, and Y is the value for a perfect match with that primer. In general, the higher the score, the higher the stability and quality of the priming at that site. A score of >200 will often give false priming results according to the Oligo 5.0 manual.

nd, not determined

effect sequence results.

T_m and percent G+C also showed less impact than expected on the dRhodamine reactions (Figure 2, C and D and Table 3). Thus, primers with low T_m and percent G+C; e.g., 37°C/28% and 44°C/28% for primer Nos. 72 and 39, and others with high T_m/percent G+C; e.g., 75°C/72% and 83°C/78% for control primer Nos. 80 and 84 and 91°C/82% for submitted primer No. 4321b, each provided sequence at well under 1% error rate (Table 3). Secondary priming potential also exhibited less of an obstacle for dRhodamine sequencing than was anticipated. Primers with very high secondary priming scores at sites within the template provided excellent sequence data. Control primer Nos. 82 and 83, and submitted primer Nos. 10Ab and 49b, had high secondary priming scores, and in each case the homology encompassed the 3′ end of the primer (Table 3). Nonetheless, these primers yielded very high-quality results.

Finally, we compared the sequencing results generated with primers selected by software to the results of manually selected primers and to the arbitrarily selected control primers. There was no observable difference in the results obtained; i.e., the average

number of errors over a 700-nt sequencing window was 2.9, 3.1 and 3.3 for all submitted primers, for submitted primers selected using software and for the control primers, respectively. Moreover, no clear trends were observed when comparing the physico-chemical characteristics of the software-designed primers to the manually designed or the control primers (data not shown).

Old Rhodamines vs. dRhodamines

The old rhodamine dye reactions were more strongly impacted by primer physico-chemical characteristics than the dRhodamine dye reactions. As described above, the old rhodamine dye reactions were consistently poorer than the dRhodamine reactions (Table 2). Moreover, the former reactions seemed more sensitive to primer characteristics. Thus, reactions with higher error rates tended to be those with lower T_m, lower percent G+C and longer homopolymeric stretches (Figure 2, panels C–E). As with the dRhodamines, the old rhodamine reactions exhibited no discernable trend in relation to secondary priming potential (Figure 2F), or primer secondary structure (data not shown).

DISCUSSION

In this study, we examined the perceptions and strategies of participant core facility personnel for the design and selection of DNA sequencing and PCR primers. The results may reveal some unexpected misconceptions in the requirements for good sequencing primer design. Thus, most laboratories espouse primer design dogma suggesting that a sequencing primer should be 18–24 nt in length with a T_m = 52°–66°C, 40%–60% G+C, 1–2-bp 3′ GC clamps and little internal structure, homopolymeric sequence or potential for secondary priming. The percent G+C and secondary priming potential were rated as the most important characteristics, but T_m, GC clamps, primer secondary and tertiary structure, length and the presence of homopolymeric runs were also thought to be relevant. Moreover, previous studies have shown that secondary structure of the template in the primer target regions can effect priming efficiency (7,8).

With regard to primer design, we were surprised to find that the majority of investigators design most of their sequencing primers manually. Thus, in spite of the perceived importance of primer characteristics and the widespread availability of primer design software, approximately 55% of the participants in this study elected to design primers manually. We believe that this observation is the result of a combination of the facts that primer design software is not yet sufficiently "user-friendly" and that the primer physico-chemical characteristics are not as important as commonly believed. It would seem that the authors of primer design software could take note of this result and streamline and enhance their products so that they are more convenient, beneficial and practical to use. Clearly, however, there are many circumstances (e.g., templates with GC-rich regions or with unusual sequences or structures, genomic sequencing or the sequencing of very large templates with many repeat regions) in which it would be advisable to use appropriate primer design software to automate and expedite primer design and synthesis.

The results of the empirical sequencing analysis were surprising in that nearly all of the primers yielded data of extremely high quality. Excellent data was obtained in spite of widely varying primer T_m and percent G+C, the presence of nearly 50% of the primer as homopolymer and the presence of very strong secondary priming sites, characteristics considered by most respondents to be the critical for sequencing primers. This study confirms earlier observations (3) that there is a broad tolerance in these and other characteristics of sequencing primers. Other factors, such as template purity or quality or technical expertise, possibly play greater roles. In this study, the plasmid template was selected for absence of sequence extension obstacles and purified by double banding in CsCl-ethidium bromide isopycnic density gradients. Therefore, this template was extremely pure and optimal for sequencing. The primers were similarly highly purified. The reactions were performed in a single high-throughput sequencing facility under tightly

controlled conditions. Different results may be obtained using less carefully purified DNA templates with unusual sequences or structures or in less rigorously controlled sequencing operations.

Finally, the data generated using old rhodamine reagents was, in general, poorer in quality than that generated using the dRhodamine reagents. Moreover, the expected trends in comparing sequence performance and primer characteristics were more prominently observed using the old rhodamine reagents. We believe it possible, if not likely, that the greater uniformity in peak height generated by the newer sequencing reagents (19) is at least partially responsible for this difference. Thus, background peaks are less likely to interfere with base calling, lessening the overall effects of potential problems like secondary priming and stutter.

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REFERENCES

1. **Adams, P.S., M.K. Dolejsi., G. Grills, S. Hardin, D.L. McMinimy, P. Morrison, J. Rush, S. Goff et al.** 1998. An analysis of techniques used to improve the accuracy of automated DNA sequencing of a GC-rich template: results from the 2nd ABRF DNA Sequence Research Group Study. J. Biomol. Techniq. *9*:9-18.
2. **Adams, P.S., M.K. Dolejsi, S. Hardin, S. Mische, B. Nanthakumar, H. Riethman, J. Rush and P. Morrisson.** 1996. DNA sequencing of a moderately difficult template: evaluation of the results from a *Thermus thermophilus* unknown test sample. BioTechniques *21*:678.
3. **Grayburn, W.S. and T.L. Sims.** 1998. Anchored oligo(dT) primers for automated dye terminator DNA sequencing. BioTechniques *25*:340-346.
4. **Hyndman, D., A. Cooper, S. Pruzinshky, D. Coad and M. Mitsuhashi.** 1996. Software to determine optimal oligonucleotide sequences based on hybridization simulation data. BioTechniques *20*:1090-1097.
5. **Ivanetich, K., L. Bibbs, R.L. Niece, N.D. Denslow, C.W. Naeve, M. Rohde and L.H. Ericsson.** 1997. Commercial biotech instrumentation survey on performance, support, service. Genetic Engineering News *17*:17.
6. **Ivanetich, K., R.L. Niece, M. Rohde, E. Fowler and T.K. Hayes.** 1993. Biotechnology core facilities: trends and update. FASEB J. *7*:1109-1114.
7. **Kaczorowski, T. and W. Szybalski.** 1996. Cooperativity of hexamer ligation. Gene *179*:189-193.
8. **Kaczorowski, T. and W. Szybalski.** 1998. Genomic DNA sequencing by SPEL-6 primer walking using hexamer ligation. Gene *223*:83-91.
9. **Lathe, R.** 1986. Synthetic oligonucleotide probes deduced from amino acid sequence data: theoretical and practical considerations. J. Mol. Biol. *183*:1-12.
10. **Maniatis, T., E.F. Fritsch and J. Sambrook.** 1982. Molecular Cloning: A Laboratory Manual. CSH Laboratory Press, Cold Spring Harbor, NY.
11. **McGraw, R.A., E.K. Steffe and S.M. Baxter.** 1990. Sequence-dependent oligonucleotide-target duplex stabilities: rules from empirical studies with a set of twenty-mers. BioTechniques *8*:674-678.
12. **Naeve, C.W., G.A. Buck, R.L. Niece, R.T. Pon, M. Robertson and A.J. Smith.** 1995. Accuracy of automated DNA sequencing: a multi-laboratory comparison of sequencing results. BioTechniques *19*:448-453.
13. **PE Biosystems.** 1991. Extension primers: recommendations for sequence selection, synthesis, and purification. *In* User Bulletin 19. PE Biosystems, Foster City, CA.
14. **Pon, R.T., G.A. Buck, K.M. Hager, C.W. Naeve, R. L. Niece, M. Robertson and A.J. Smith.** 1996. Multi-facility survey of oligonucleotide synthesis and an examination of the performance of unpurified primers in automated DNA sequencing. BioTechniques *21*:680-685.
15. **Pon, R.T., G.A. Buck, R.L. Niece, M. Robertson, A.J. Smith and E. Spicer.** 1994. A survey of nucleic acid services in core laboratories. BioTechniques *17*:526-534.
16. **Rychlik, W. and R.E. Rhoads.** 1989. A computer program for choosing optimal oligonucleotides for filter hybridization, sequencing and in vitro amplification of DNA. Nucleic Acids Res. *17*:8543-8551.
17. **Strauss, E.C., J.A. Kobori, G. Siu and L.E. Hood.** 1986. Specific-primer-directed DNA sequencing. Anal. Biochem. *154*:353-360.
18. **Voss, H., S. Wiemann, D. Grothues, C. Sensen, J. Zimmermann, C. Schwager, J. Stegemann, H. Erfle, T. Rupp and W. Ansorge.** 1993. Automated low-redundancy large-scale DNA sequencing by primer walking. BioTechniques *15*:714-721.
19. **Zakeri, H., G. Amparo, S.-M. Chen, S. Spurgeon and P.-Y. Kwok.** 1998. Peak height pattern in dichloro-rhodamine and energy transfer dye terminator sequencing. BioTechniques *25*:406-414.

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Continuous Fluorescence Monitoring of Rapid Cycle DNA Amplification

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ABSTRACT

Rapid cycle DNA amplification was continuously monitored by three different fluorescence techniques. Fluorescence was monitored by (i) *the double-strand-specific dye SYBR® Green I,* (ii) *a decrease in fluorescein quenching by rhodamine after exonuclease cleavage of a dual-labeled hydrolysis probe and* (iii) *resonance energy transfer of fluorescein to Cy5™ by adjacent hybridization probes. Fluorescence data acquired once per cycle provides rapid absolute quantification of initial template copy number. The sensitivity of SYBR Green I detection is limited by nonspecific product formation. Use of a single exonuclease hydrolysis probe or two adjacent hybridization probes offers increasing levels of specificity. In contrast to fluorescence measurement once per cycle, continuous monitoring throughout each cycle monitors the temperature dependence of fluorescence. The cumulative, irreversible signal of hydrolysis probes can be distinguished easily from the temperature-dependent, reversible signal of hybridization probes. By using SYBR Green I, product denaturation, annealing and extension can be followed within each cycle. Substantial product-to-product annealing occurs during later amplification cycles, suggesting that product annealing is a major cause of the plateau effect. Continuous within-cycle monitoring allows rapid optimization of amplification conditions and should be particularly useful in developing new, standardized clinical assays.*

INTRODUCTION

Fluorescent probes can be used to detect and monitor in vitro DNA amplification (14). Useful probes include double-stranded DNA (dsDNA)-specific dyes and sequence-specific probes. With the intercalater ethidium bromide and ultraviolet illumination, red fluorescence increases after amplification in microcentrifuge tubes (3) or capillaries (22). Sequence-specific fluorescence detection is possible with oligonucleotide probes. For example, dual-labeled fluorescein/rhodamine probes may be cleaved during polymerase extension by 5′-exonuclease activity, separating the fluorophores and increasing the fluorescein/rhodamine fluorescence ratio (5,8,9). Alternately, "molecular beacons" have been described with a fluorogenic conformational change when hybridized to their targets (7).

Fluorescence can be measured after temperature cycling is complete, once per cycle as a monitor of product accumulation or continuously within each cycle. Sequence-specific methods (7–9) have been limited in the past to endpoint analysis. The potential of once-per-cycle monitoring for quantification of initial template copy number was first suggested and developed by Higuchi et al. using ethidium bromide (3,4). Fluorescence is acquired during the extension or combined annealing/extension phase of each cycle and is related to product concentration. A quantitative assay for hepatitis C RNA using the intercalater YO-PRO-1™ has been reported (6). To date, continuous monitoring of fluorescence within each cycle has found little use. Higuchi et al. continuously monitored amplification during 4-min temperature cycles using a 10-s integration time (3). An inverse correlation of ethidium fluorescence to temperature was noted, with product accumulation resulting in increased fluorescence during annealing/extension.

If fluorescence is continuously monitored within each temperature cycle, the hybridization of amplification products and probes can be followed during amplification. With dsDNA dyes, product denaturation and reannealing can be monitored. With probes that change fluorescence upon hybridization, probe melting temperatures can be determined. With rapid, homogeneous control of sample temperature, the kinetics of hybridization can be followed. We have previously used capillaries and forced-air heating for precise temperature control that allows 30 cycles in less than 15 min (18–22). By minimizing denaturation and annealing times, the specificity and yield of such "rapid cycle" amplifications are also improved (2,12,15,16,20–22). In addition to facilitating rapid heat transfer, glass capillaries are optically clear and make natural cuvettes for fluorescence analysis.

Three different fluorescence techniques for following rapid cycle DNA amplification are studied here using instrumentation described elsewhere (23). Instead of ethidium bromide, SYBR® Green I is used as a dsDNA-specific dye. A 5′-exonuclease probe is then compared to SYBR Green I monitoring. Finally, a novel fluorescence scheme based on adjacent hybridization probes with resonance energy transfer from fluorescein to the cyanine dye Cy5™ (11) is demonstrated. Once-per-cycle monitoring of multiple samples is a powerful quantitative tool. Continuous monitoring within the temperature cycles of DNA amplification can reveal the mechanism of probe fluorescence, rapidly optimize tempera-

ture/time conditions and potentially even control temperature cycling parameters. The melting and annealing of products and probes can be followed during amplification.

MATERIALS AND METHODS

DNA amplification was performed in 50 mM Tris-HCl, pH 8.5 (25°C), 3 mM $MgCl_2$, 500 µg/mL bovine serum albumin, 0.5 µM of each primer, 0.2 mM of each deoxyribonucleoside triphosphate and 0.2 U of *Taq* DNA polymerase per 5-µL sample, unless otherwise stated. Human genomic DNA (denatured for 1 min by boiling) or purified amplification product was used as DNA template. Purified amplification product was obtained by phenol /chloroform extraction and ethanol precipitation (17), followed by removal of primers by repeated washing through a Centricon® 30 microconcentrator (Amicon, Beverly, MA, USA). Template concentrations were determined by absorbance at 260 nm. A_{260}/A_{280} ratios of templates were greater than 1.7.

Primers were synthesized by standard phosphoramidite chemistry (Gene Assembler® Plus; Pharmacia Biotech, Piscataway, NJ, USA). SYBR Green I was obtained from Molecular Probes (Eugene, OR, USA). The β-actin primers and fluorescein/rhodamine dual probe were obtained from Perkin-Elmer (Norwalk, CT, USA). The human β-globin primers RS42/KM29 (536 bp) and PC03/PC04 (110 bp) have been described previously (19). The single-labeled probes 5′-CAAACAG-ACACCATGGTGCACCTGACTCC-TGAGGA-fluorescein-3′ and 5′-Cy5-AAGTCTGCCGTTACTGCCCTGTG-GGGCAAG-phosphate-3′ were synthesized using a fluorescein phosphoramidite (Glen Research, Sterling, VA, USA), a Cy5™ phosphoramidite (Pharmacia Biotech) and a chemical phosphorylation reagent (Glen Research). These adjacent probes hybridize internal to the PC03/PC04 β-globin primer pair on the same DNA strand and are separated by one base pair. Probes were purified by reverse-phase C-18 high-pressure liquid chromatography, and homogeneity was checked by polyacrylamide gel electrophoresis and absorbance (A_{260} and the absorbance maximum of the fluorophore). The β-actin hydrolysis probe and the β-globin hybridization probes were used at 0.2 µM each. Figure 1 schematically compares the differences between the three fluorescence monitoring techniques: ds-DNA-specific dyes, hydrolysis probes and hybridization probes.

Amplification samples of 5 µL were loaded into glass capillary tubes (1.02 mm o.d., 0.56 mm i.d.) and sealed. The tubes were cleaned with optical-grade methanol and then loaded into the carousel of a fluorescence temperature cycler described elsewhere (23). For continuous monitoring of a single sample, the carousel was positioned at maximal fluorescence and signals acquired every 200 ms. For multiple tubes, signals were obtained once each cycle by sequentially positioning the carousel at each tube for 100 ms.

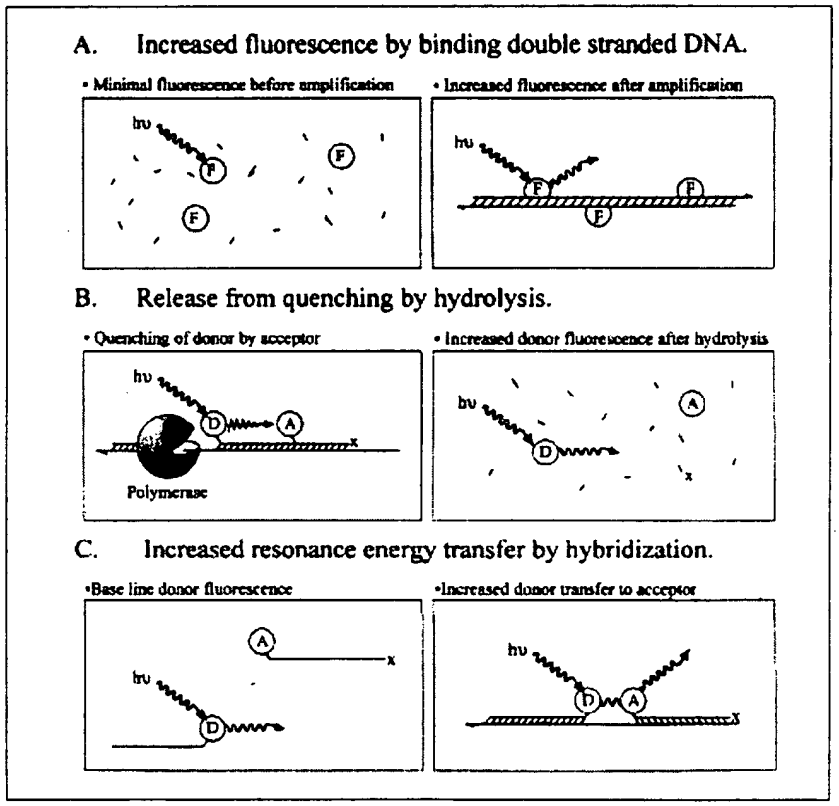

Figure 1. Schematic diagram comparing three different fluorescence-monitoring systems for DNA amplification. System A uses dsDNA-specific dyes (F) such as SYBR Green I, which increase in fluorescence when bound to accumulating amplification product. System B uses dual-labeled probes (8,9) and depends on the 5′-exonuclease activity of the polymerase to separate donor (D) and acceptor (A) by hydrolysis. Donor fluorescence is increased by removing acceptor quenching. System C depends on the independent hybridization of adjacent donor (D) and acceptor (A) probes. Their approximation increases resonance energy transfer from the donor to the acceptor. Other symbols are "hυ" for excitation light and "x" for a 3′-phosphate.

RESULTS

Figure 1 illustrates the three different fluorescence techniques used for continuous monitoring of DNA amplification. Figures 2, 3 and 4 demonstrate the application of these techniques for initial template quantification by fluorescence monitoring once each cycle. In Figure 2, the fluorescence of the ds-DNA-specific dye SYBR Green I is followed. A 10^7–10^8 range of initial template concentration can be discerned. When the data are normalized as the percent maximal fluorescence of each tube, 100 initial copies are clearly separated from 10 copies. However, the difference between 1 and 10 copies is marginal, and no difference is observed

between 0 and 1 average copies per tube. Nonspecific detection of undesired products after many cycles is a limitation of fluorescence monitoring of dsDNA.

Specific fluorescence monitoring can be obtained with sequence-specific fluorescent probes. In Figure 3, amplification is monitored using a dual-labeled hydrolysis probe. The fluorescence signal is expressed as a ratio of fluorescein to rhodamine fluorescence. Signal generation with 5′-exonuclease probes is dependent not only on DNA synthesis, but requires hybridization and hydrolysis between the fluorophores of the dual-labeled probe. Hydrolysis reduces quenching of fluorescein, and the fluorescence ratio of fluorescein-to-rhodamine emission increases. Whereas the fluorescence from dsDNA-specific dyes plateaus with excess cycling, the signal from hydrolysis probes continues to increase after many cycles. Even though no net product is being synthesized, probe hybridization and hydrolysis continue to occur. In contrast to dsDNA dyes, no fluorescence signal is generated in the absence of template.

In Figure 4, amplification is monitored using adjacent hybridization probes and is expressed as a ratio of Cy5 to fluorescein fluorescence. One of the probes is labeled 3′ with fluorescein, and the other probe is labeled 5′ with Cy5. When hybridized to accumulating product, the probes are separated by a 1-bp gap, and the Cy5-to-fluorescein fluorescence ratio increases. The change in fluorescence ratio during hybridization is largely due to an increase in Cy5 fluorescence from resonance energy transfer (data not shown). In contrast to hydrolysis probes, the fluorescence signal of hybridization probes tends to decrease with excessive cycling. No fluorescence signal is generated in the absence of template.

Sequence-specific probes have an even greater dynamic range for template quantification than do dsDNA dyes. As the template copy number decreases below 10^3, signal intensity decreases because specific amplification efficiency decreases, but low copy numbers can still be quantified because the negative control signal is stable (Figures 3 and 4). Although multiple samples would need to be run to confirm Poisson statistics, it appears that these specific techniques can discriminate a single initial template copy from negative controls (compare 0 and 1 average initial template copies in Figures 3 and 4).

With each technique, the fluorescence response is not strictly proportional to the amount of specific product. With SYBR Green I, two factors contribute to this nonlinearity. First,

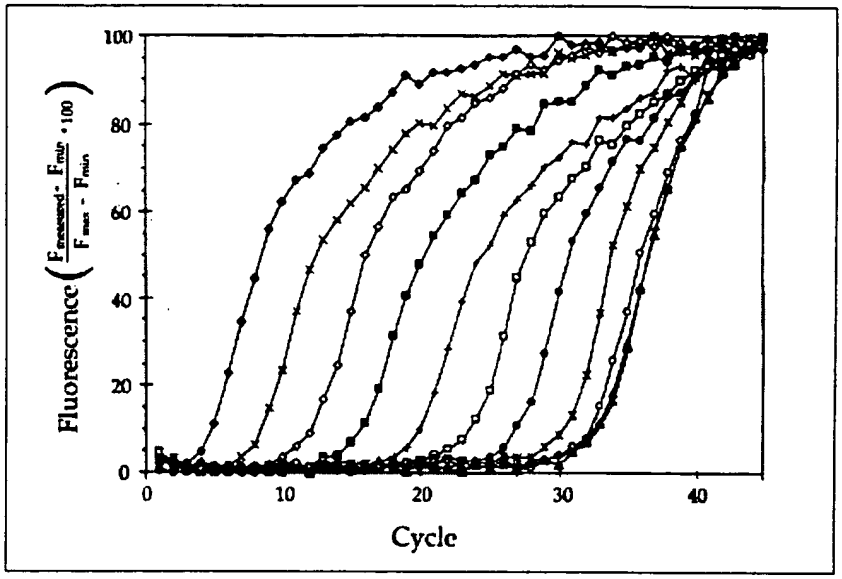

Figure 2. Fluorescence vs. cycle number plot of DNA amplification monitored with the dsDNA-specific dye SYBR Green I. A 536-bp fragment of the human β-globin gene was amplified from 10^9 (◆), 10^8 (×), 10^7 (◇), 10^6 (■), 10^5 (+), 10^4 (□), 10^3 (●), 10^2 (*), 10 (O), 1 (▲) and 0 (—) average template copies in the presence of a 1:10 000 dilution of SYBR Green I. Each temperature cycle was 28 s long (95°C maximum, 61°C minimum, 15 s at 72°C, average rate between temperatures 5.2°C/s) and 45 cycles were completed in 21 min. All samples were amplified simultaneously and monitored for 100 ms between seconds 5 and 10 of the extension phase. The data are normalized as a percentage of the difference between minimum and maximum values for each tube (y-axis).

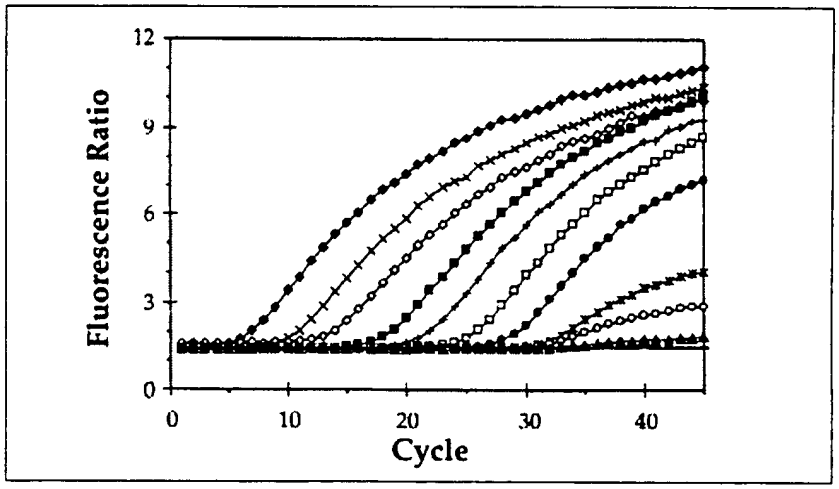

Figure 3. Fluorescence ratio (fluorescein/rhodamine) vs. cycle number plot of DNA amplification monitored with a dual-labeled 5′-exonuclease hydrolysis probe. A 295-bp fragment of the human β-actin gene was amplified from 10^9 (◆), 10^8 (×), 10^7 (◇), 10^6 (■), 10^5 (+), 10^4 (□), 10^3 (●), 10^2 (*), 10 (O), 1 (▲) and 0 (—) average template copies in the presence of 0.2 μM of the hydrolysis probe. Each temperature cycle was 26 s long (94°C maximum, 60°C for 15 s, average rate between temperatures 6.2°C/s) and 45 cycles were completed in under 20 min. All samples were amplified simultaneously and monitored for 100 ms between seconds 5 and 10 of the annealing/extension phase.

nonspecific amplification of alternative templates results in fluorescence unrelated to specific product, particularly after many cycles (Figure 2). In addition, when the fluorescence of purified DNA standards is measured, the response is only linear to 10–20 ng of DNA per 5-µL reaction under the conditions used (data not shown). Higher concentrations of DNA show proportionally less fluorescence, presumably because the amount of SYBR Green I becomes limiting. Higher concentrations of SYBR Green I than those used here (<1:7000 dilution) inhibit amplification (data not shown). With hydrolysis probes, the fluorescence signal continues to increase after the plateau phase has been reached (Figure 3). With hybridization probes, the fluorescence decreases during the plateau phase (Figure 4). Despite the nonlinearity of these fluorescence techniques, they are very useful for absolute quantification of initial template copy number when fluorescence is measured for each amplification cycle (Figures 2–4).

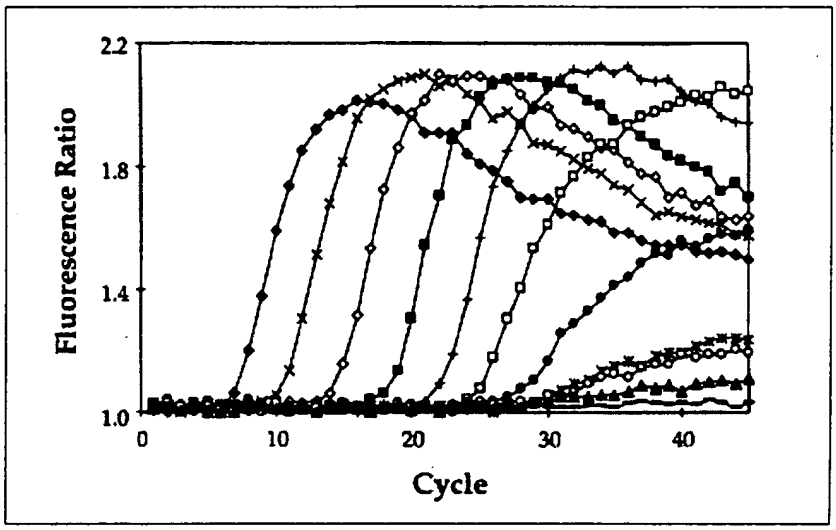

Figure 4. Fluorescence ratio (Cy5/fluorescein) vs. cycle number plot of DNA amplification monitored with 3′-fluorescein and 5′-Cy5 adjacent hybridization probes. A 110-bp fragment of the human β-globin gene was amplified from 10^9 (◆), 10^8 (×), 10^7 (◇), 10^6 (■), 10^5 (+), 10^4 (□), 10^3 (●), 10^2 (*), 10 (O), 1 (▲) and 0 (—) average template copies in the presence of 0.2 µM of each hybridization probe. Each temperature cycle was 30 s long (94°C maximum, 59°C for 20 s, average rate between temperatures 7.0°C/s) and 45 cycles were completed in under 23 min. All samples were amplified simultaneously and monitored for 100 ms between seconds 12 and 17 of the annealing/extension phase.

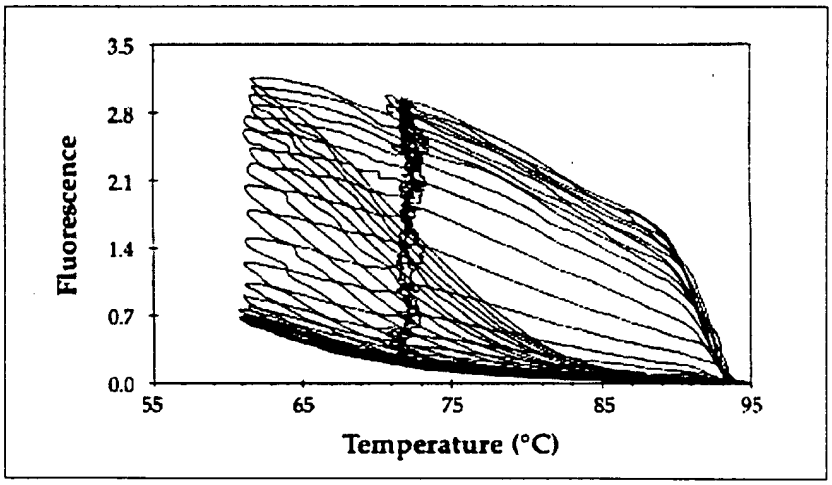

Figure 5. Continuous fluorescence vs. temperature plot of DNA amplification with the dsDNA-specific dye SYBR Green I. A 536-bp fragment of the human β-globin gene was amplified from 25 ng of genomic DNA and a 1:10 000 dilution of SYBR Green I. Temperature cycling conditions are given in Figure 2. Cycles 15–40 are shown. Product denaturation is evident between 90° and 93°C, and significant product-to-product annealing is observed during cooling.

Because fluorescence depends on temperature, fluorescence is usually acquired only once per cycle at a constant temperature to monitor product yield. This eliminates any confounding effect of temperature on fluorescence. However, interesting and useful information about hybridization can be obtained by monitoring fluorescence continuously throughout each temperature cycle. For example, fluorescence vs. temperature plots of amplification with SYBR Green I show the temperature dependence of strand status during DNA amplification (Figure 5). Early cycles appear identical, with a nonlinear increase in fluorescence at lower temperatures. As amplification proceeds, later cycles appear as rising loops between annealing and denaturation temperatures that show significant hysteresis. By hysteresis, we mean that the observed fluorescence during heating is greater than that during cooling. As the sample is heated, fluorescence is high until denaturation occurs (apparent as a sharp drop in fluorescence). As the sample cools from denaturation to annealing temperatures, fluorescence increases rapidly, apparently reflecting product-to-product annealing. Fluorescence also increases during extension while the temperature is held constant.

Fluorescence vs. temperature plots of 5′-exonuclease probes confirm that probe hydrolysis (not hybridization) is the mechanism of signal generation. In Figure 6, a fluorescence vs. temperature plot is shown for amplification with the β-actin exonuclease probe. During each cycle, the fluorescence ratio varies linearly with temperature and there is little, if any, hysteresis. Although the fluorescence of both fluorescein and rhodamine decreases with increasing temperature (data not shown), the rate of change is greater for rhodamine, resulting in an increasing ratio with increasing temperature. The fluorescence ratio increases during the annealing/extension phase at a constant temperature when probe hydrolysis is

presumed to occur. In contrast, with adjacent hybridization probes, the fluorescence signal is dependent only on hybridization, not hydrolysis. Fluorescence vs. temperature plots of amplification with hybridization probes show obvious hysteresis (Figure 7). During heating to product denaturation temperatures, the probes appear to dissociate between 65° and 75°C, returning the fluorescence ratio to background levels. These temperature-dependent hybridization effects are not apparent with the 5′-exonuclease probe (Figure 6).

DISCUSSION

Three different chemistries for fluorescent monitoring of DNA amplification have been studied: a dsDNA-specific dye, a dual-labeled exonuclease hydrolysis probe and adjacent hybridization probes. The hydrolysis and hybridization probes are sequence specific. Hydrolysis probes require one hybridization event for signal generation, whereas hybridization probes require two independent hybridizations. Figure 2 schematically compares and contrasts the three fluorescence monitoring systems.

dsDNA-specific dyes such as ethidium bromide (3,4,22) or SYBR Green I can be used as generic indicators of amplification. We used SYBR Green I instead of ethidium bromide because it has an excitation maximum near fluorescein and, in our hands, gave a stronger signal with DNA than excitation of ethidium bromide with visible light. These dyes depend on the specificity inherent in the amplification primers. Currently, nonspecific amplification after many cycles limits detection sensitivity to about 100 initial template copies (Figure 2, see also Reference 4). Improvements in amplification specificity could remove this limitation.

When low-copy-number detection and quantification are needed, additional specificity can be provided by sequence-specific fluorescent probes. Hydrolysis of a dual-labeled exonuclease probe is sequence specific (8,9). However, the design, synthesis and purification of dual-labeled hydrolysis probes require care. Hybridization is a necessary but not sufficient condition for hydrolysis; all probes are not cleaved efficiently. Synthesis of the dual-labeled probes involves manual addition of the rhodamine label, and at least one stage of high-pressure liquid chromatography is required for purification. In addition, the signal generated by exonuclease probes is cumulative and only indirectly related to product concentration. Hence, the fluorescence signal continues to increase even after the amount of product has reached a plateau (Figures 3 and 6).

Instead of depending on hydrolysis

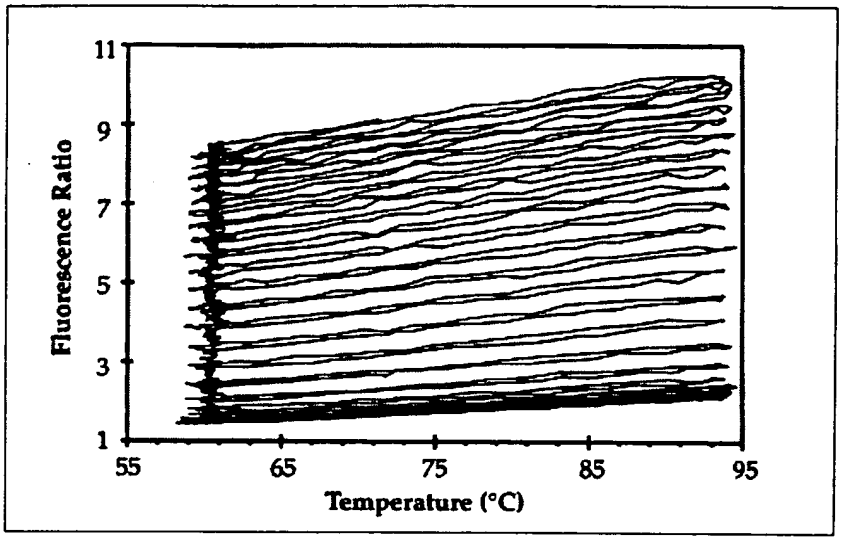

Figure 6. Continuous fluorescence (fluorescein/rhodamine) vs. temperature plot of DNA amplification monitored with a dual-labeled 5′-exonuclease hydrolysis probe. A 295-bp fragment of the human β-actin gene was amplified from 25 ng of genomic DNA. Temperature cycling conditions are given in Figure 3. Cycles 20–45 are shown. The dependence of fluorescence on temperature is linear each cycle, and no hybridization effect is apparent.

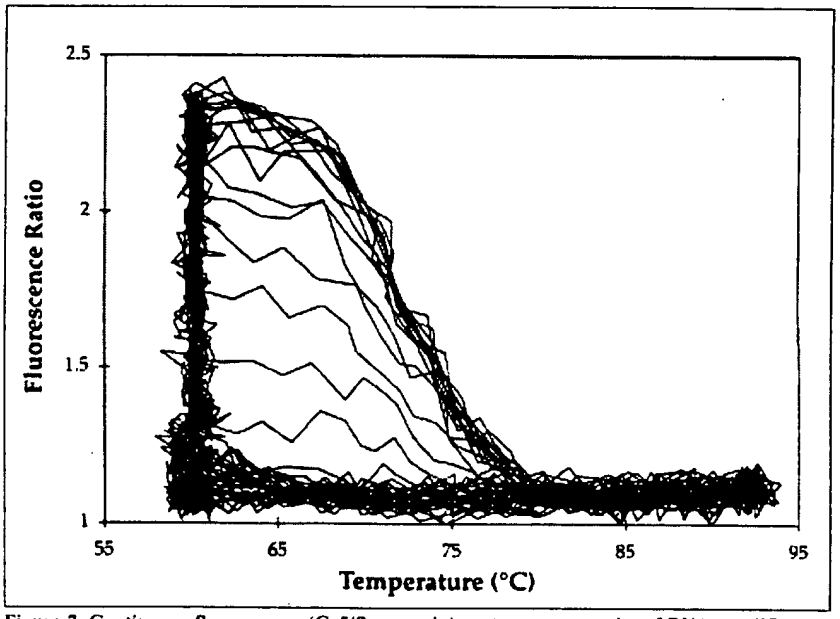

Figure 7. Continuous fluorescence (Cy5/fluorescein) vs. temperature plot of DNA amplification monitored with adjacent hybridization probes. A 110-bp fragment of the human β-globin gene was amplified from 25 ng of genomic DNA. Temperature cycling conditions are given in Figure 4. Cycles 20–40 are shown. The hybridization probes appear to melt off the target DNA sequence each cycle between 65° and 75°C.

of a dual-labeled probe, hybridization can be detected directly through resonance energy transfer as outlined by Morrison (10). By using two adjacent probes labeled separately with fluorescein and Cy5, energy transfer to Cy5 increases with product accumulation (Figures 4 and 7). In contrast to exonuclease probes, probe synthesis is relatively simple because amidites for both fluorescein and Cy5 are available for direct incorporation during automated synthesis. Signal generation requires two independent hybridization events to occur each cycle and is more directly related to product concentration than cumulative hydrolysis probes. However, after many cycles, the fluorescence from hybridization probes decreases (Figure 4), possibly because of probe consumption by exonuclease hydrolysis.

We are not aware of prior reports using fluorescein and Cy5 as a resonance energy transfer pair, although phycoerythrin and Cy7™ (Amersham International, Little Chalfont, Bucks, England, UK) (with similar spectral separation) have been used as a bright tandem dye in immunofluorescence (13). With fluorescein and Cy5, the spectral overlap is small, but the molar absorption coefficient and absorption wavelengths of Cy5 are high. All three factors (overlap, absorptivity and wavelength) contribute to the overlap integral that determines energy transfer rates (24). Cy5 also has low absorbance at fluorescein excitation wavelengths (11), reducing direct excitation of the acceptor.

Many aspects of hydrolysis and hybridization probes remain to be studied. The effects of probe length, melting temperature and concentration, distance between hybridization probes, distance to primers, temperature profiles, acquisition point within a cycle and type of polymerase have not been systematically optimized.

DNA amplification is extensively used but not rigorously understood. Continuous fluorescence monitoring provides an instantaneous window into the amplification process. For example, product denaturation occurs in less than 1 s (20,22), yet most protocols call for 10 s to 1 min of denaturation. By monitoring with dsDNA-specific dyes, product denaturation can be observed during each amplification cycle (Figure 5), a convincing demonstration that most denaturation protocols are excessive. To give another example, many causes of the "plateau effect" have been proposed, but little data are available to distinguish between alternatives. Figure 5 shows that product-to-product annealing is very rapid. In fact, during later cycles of amplification, a majority of product anneals to itself each cycle during cooling before the primer annealing temperature has been reached. This rapid reannealing is observed with cooling rates of 5°–10°C/s, characteristic of rapid cycling. Product reannealing with slower, conventional temperature cyclers would be greater. Product-to-product annealing appears to be a major, and perhaps the sole, cause of the "plateau effect".

As previously suggested (22), continuous fluorescence monitoring within each temperature cycle can be used to control temperature cycling parameters. With dsDNA-specific dyes, amplification can be stopped after a certain amount of product is synthesized, thus avoiding overamplification of alternative templates. The extension phase of each cycle needs to be continued only as long as fluorescence increases. Product denaturation can be assured each cycle by increasing the temperature until the fluorescence reaches baseline. This kind of fluorescence feedback should allow very rapid optimization of new assays. Limiting the time that product is exposed to denaturation temperatures may also be useful for the amplification of long products (1,2).

Additional uses of continuous monitoring with fluorescent dyes can be envisioned. For example, with fine temperature control and dsDNA-specific dyes, product purity could be estimated by melting curves. With rapid temperature control, absolute product concentration could be determined by product-to-product annealing kinetics. The only requirements are fluorescence monitoring, the ability to change temperatures rapidly and strict intra-sample temperature homogeneity. Aspects of instrument design are discussed elsewhere (23).

Conventional end-point analysis of DNA amplification by gel electro-

phoresis identifies product size and estimates purity. However, because amplification is at first stochastic, then exponential, and finally stagnant, the utility of end-point analysis is limited for quantification. Fluorescence monitoring every cycle during DNA amplification is an extraordinarily powerful technique for quantification. With simple instrumentation and fluorescent monitoring each cycle, sequence-specific detection and quantification can be achieved in 5–20 min after temperature cycling has begun. Although the final fluorescence signal is decreased when low copy numbers are amplified, quantification between 0 and 1000 initial template copies appears possible (Figures 3 and 4). These techniques should be particularly useful in assays where rapid quantification is desired, such as in the amplification of clinical serum viruses.

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REFERENCES

1. **Barnes, W.M.** 1994. PCR amplification of up to 35-kb DNA with high fidelity and high yield from lambda bacteriophage templates. Proc. Natl. Acad. Sci. USA *91*:2216-2220.
2. **Gustafson, C.E., R.A. Alm and T.J. Trust.** 1993. Effect of heat denaturation of target DNA on the PCR amplification. Gene *123*:241-244.
3. **Higuchi, R., G. Dollinger, P.S. Walsh and R. Griffith.** 1992. Simultaneous amplification and detection of specific DNA sequences. Bio/Technology *10*:413-417.
4. **Higuchi, R., C. Fockler, G. Dollinger and R. Watson.** 1993. Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. Bio/Technology *11*:1026-1030.
5. **Holland, P.M., R.D. Abramson, R. Watson and D.H. Gelfand.** 1991. Detection of specific polymerase chain reaction product by utilizing the 5′ to 3′ exonuclease activity of Thermus aquaticus DNA polymerase. Proc. Natl. Acad. Sci. USA *88*:7276-7280.
6. **Ishiguro, T., J. Saitch, H. Yawata, H. Yamagishi, S. Iwasaki and Y. Mitoma.** 1995. Homogeneous quantitative assay of hepatitis C virus RNA by polymerase chain reaction in the presence of a fluorescent intercalater. Anal. Biochem. *229*:207-213.
7. **Kramer, R.K. and S. Tyagi.** 1996. Molecular beacons: probes that fluoresce upon hybridization. Nature Biotech. *14*:303-308.
8. **Lee, L.G., C.R. Connell and W. Bloch.** 1993. Allelic discrimination by nick-translation PCR with fluorogenic probes. Nucleic Acids Res. *21*:3761-3766.
9. **Livak, K.J., S.J.A. Flood, J. Marmaro, W. Giusti and K. Deetz.** 1995. Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. PCR Methods Appl. *4*:357-362.
10. **Morrison, L.E.** 1992. Detection of energy transfer and fluorescence quenching, p. 311-352. *In* L.J. Kricka (Ed.), Nonisotopic DNA Probe Techniques. Academic Press, San Diego.
11. **Mujumdar, R.B., L.A. Ernst, S.R. Mujumdar and A.S. Waggoner.** 1989. Cyanine dye labeling reagents containing isothiocyanate groups. Cytometry *10*:11-19.
12. **Odelberg, S.J. and R. White.** 1993. A method for accurate amplification of polymorphic CA-repeat sequences. PCR Methods Appl. *3*:7-12.
13. **Roederer, M., A.B. Kantor, D.R. Parks and L.A. Herzenberg.** 1996. Cy7PE and Cy7APC: bright new probes for immunofluorescence. Cytometry *24*:191-197.
14. **Saiki, R.K., D.H. Gelfand, S. Stoffel, S.J. Scharf, R. Higuchi, G.T. Horn, K.B. Mullis and H.A. Erlich.** 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science *239*:487-491.
15. **Swerdlow, H., K. Dew-Jager and R.F. Gesteland.** 1993. Rapid cycle sequencing in an air thermal cycler. BioTechniques *15*:512-519.
16. **Tan, S.T. and J.H. Weis.** 1992. Development of a sensitive reverse transcriptase PCR assay, RT-PCR, utilizing rapid cycle times. PCR Methods Appl. *2*:137-143.
17. **Wallace, D.M.** 1987. Large- and small-scale phenol extractions and precipitation of nucleic acids, p. 33-48. *In* S.L. Berger and A.R. Kimmel (Eds.), Guide to Molecular Cloning Techniques (Methods in Enzymology, Vol. 152). Academic Press, Orlando.
18. **Wittwer, C.T., G.C. Fillmore and D.J. Garling.** 1990. Minimizing the time required for DNA amplification by efficient heat transfer to small samples. Anal. Biochem. *186*:328-331.
19. **Wittwer, C.T., G.C. Fillmore and D.R. Hillyard.** 1989. Automated polymerase chain reaction in capillary tubes with hot air. Nucleic Acids Res. *17*:4353-4357.
20. **Wittwer, C.T. and D.J. Garling.** 1991. Rapid cycle DNA amplification: time and temperature optimization. BioTechniques *10*:76-83.
21. **Wittwer, C.T., B.C. Marshall, G.B. Reed and J.L. Cherry.** 1993. Rapid cycle allele-specific amplification: studies with the cystic fibrosis delta F508 locus. Clin. Chem. *39*:804-809.
22. **Wittwer, C.T., G.B. Reed and K.M. Ririe.** 1994. Rapid cycle DNA amplification, p. 174-181. *In* K.B. Mullis, F. Ferre and R.A. Gibbs (Eds.), The Polymerase Chain Reaction. Birkhauser, Boston.
23. **Wittwer, C.T., K.M. Ririe, R.V. Andrew, D.A. David, R.A. Gundry and U.J. Balis.** 1997. The LightCycler™: a microvolume multisample fluorimeter with rapid temperature control. BioTechniques *22*:176-181.
24. **Wu, P. and L. Brand.** 1994. Resonance energy transfer: methods and applications. Anal. Biochem. *218*:1-13.

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Utilization of the *rpoB* Gene as a Specific Chromosomal Marker for Real-Time PCR Detection of *Bacillus anthracis*

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The potential use of *Bacillus anthracis* as a weapon of mass destruction poses a threat to humans, domesticated animals, and wildlife and necessitates the need for a rapid and highly specific detection assay. We have developed a real-time PCR-based assay for the specific detection of *B. anthracis* by taking advantage of the unique nucleotide sequence of the *B. anthracis rpoB* gene. Variable region 1 of the *rpoB* gene was sequenced from 36 *Bacillus* strains, including 16 *B. anthracis* strains and 20 other related bacilli, and four nucleotides specific for *B. anthracis* were identified. PCR primers were selected so that two *B. anthracis*-specific nucleotides were at their 3′ ends, whereas the remaining bases were specific to the probe region. This format permitted the PCR reactions to be performed on a LightCycler via fluorescence resonance energy transfer (FRET). The assay was found to be specific for 144 *B. anthracis* strains from different geographical locations and did not cross-react with other related bacilli (175 strains), with the exception of one strain. The PCR assay can be performed on isolated DNA as well as crude vegetative cell lysates in less than 1 h. Therefore, the *rpoB*-FRET assay could be used as a new chromosomal marker for rapid detection of *B. anthracis*.

Bacillus anthracis is a causal agent of anthrax, a serious and often fatal infection of livestock and humans. It is considered one of the most effective biological weapons of mass destruction because of its highly pathogenic nature and spore-forming capability and has attracted attention due to its potential use as a biological warfare agent (2). This bacterium can infect humans by cutaneous, gastrointestinal, or respiratory routes. The standard laboratory method of identification takes advantage of the lytic nature of the *B. anthracis*-specific gamma bacteriophage (9). Anthrax bacilli are often distinguished on the basis of time-consuming morphological or phenotypic characteristics, such as gram-positive staining, spore-forming capability, nonhemolytic reaction on sheep blood agar, sensitivity to penicillin, nonmotile nature, and inability to ferment salicin (11). *B. anthracis* is distinguished from the other members of the closely related *Bacillus cereus* group of bacteria by the presence of the toxin-encoding pXO1 (19, 24) and capsule-encoding pXO2 plasmids (14, 23, 34). Both plasmids are needed for virulence; thus, the absence of either plasmid results in attenuation.

B. anthracis, *Bacillus thuringiensis*, *B. cereus*, and *Bacillus mycoides*, are members of the *B. cereus* group of bacilli. These closely related bacteria are pathogens of mammals (*B. anthracis* and *B. cereus*) and insects (*B. thuringiensis*). The *B. cereus* group is one of the most taxonomically ambiguous group of bacilli (27). In fact, DNA-DNA hybridization (30) and pulsed-field gel electrophoresis (15) have shown great homology among *B. anthracis*, *B. thuringiensis*, and *B. cereus*. A recent multilocus enzyme electrophoresis study has concluded that the members of this group belong to one species (16).

Although specific assays are available for the detection of pathogenicity-related plasmids (18, 28), chromosomal markers in conjuction with plasmid markers should be used for complete genotyping of *B. anthracis* strains. Such a combined approach will provide insight into the chromosomal backbone or genetic background and indicate the pathogenic nature of the strain. Plasmids are more unstable than chromosomal DNA, and isolates lacking either or both plasmids have been found to exist in nature (33). Also, pXO2 has been successfully transferred into other bacilli, and toxin genes, such as *lef* and *cya*, have been expressed in heterologous systems (4, 5, 20). Thus, naturally occurring as well as genetically modified *B. anthracis* strains cannot be characterized without ambiguity. Moreover, chromosomal markers are stable targets for detection and are important for accurate identification of *B. anthracis* in outbreaks (26) as well as during the analysis of ancient samples (C. Redmond, M. J. Pearce, R. J. Manchee, and B. P. Berdal, Letter, Nature 393:747–748).

Several chromosomal markers are currently available for *B. anthracis* detection, such as the *vrrA* gene (1, 17), Ba813 marker (25), and SG-850 marker (10). These marker assays suffer from being time consuming or labor intensive or having limited specificity. For instance, the SG-850 assay involves PCR amplification of the SG-749 locus, followed by enzymatic digestion with *AluI* and gel analysis. The *vrrA* marker can group *B. anthracis* isolates into several categories based on the number of repeat units of this sequence, which requires post-PCR analysis (17). Recently some *B. cereus* and *B. thuringiensis* isolates have been found to contain the Ba813 marker (26); hence its use as a *B. anthracis*-specific target is questionable (29). The 16S rRNA gene also does not provide sufficient polymorphism to differentiate *B. anthracis* from closely related

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TABLE 1. *B. anthracis* strains used in the *rpoB*-FRET PCR assays

Strain	pXO1[a]	pXO2[a]	Origin	Strain	pXO1[a]	pXO2[a]	Origin
7700	−	−	Africa	A66	+	+	Europe
7702	+	−	Africa	A67	+	+	Europe
Sterne	+	−	Africa	A68	+	+	Europe
AC1	+	−	S. America	A69	+	+	Europe
AC2	+	−	S. America	A72	+	+	Europe
AC3	+	+	S. America	A73	−	+	Europe
Texas 0077	+	−	N. America	A74	−	−	Europe
Ames	+	+	N. America	001	+	+	Asia
Δ Ames	−	−	N. America	002	+	+	Asia
Vollum	+	+	Europe	044	+	−	Asia
4229	−	+	Europe	103	+	+	Asia
RA3	+	+	Europe	104	+	+	Asia
RA3R	+	−	Europe	105	+	+	Asia
RA4	+	+	Europe	108	+	+	Asia
7611	+	+	Europe	116	+	+	Asia
7611R	+	−	Europe	125	+	−	Asia
10517	+	+	Europe	126	+	+	Asia
6183	+	+	Europe	128	+	−	Asia
6042	+	+	Europe	129	+	+	Asia
BC 575	+	+	Europe	136	+	+	Asia
9066	+	+	Europe	137	+	+	Asia
7193	+	+	Europe	139	+	+	Asia
6681	+	+	Europe	140	+	+	Asia
8403	+	+	Europe	141	+	+	Asia
9240	+	+	Europe	142	+	−	Asia
8490	+	+	Europe	143	+	−	Asia
6291	+	+	Europe	144	+	+	Asia
A3	−	+	Europe	145	+	+	Asia
A4	−	+	Europe	146	+	+	Asia
A5	+	+	Europe	147	+	+	Asia
A6	−	+	Europe	148	+	+	Asia
A7	+	+	Europe	149	+	+	Asia
A8	+	+	Europe	150	+	+	Asia
A9	+	+	Europe	151	+	+	Asia
A10	+	+	Europe	152	+	+	Asia
A11	+	+	Europe	153	+	+	Asia
A12	+	+	Europe	154	+	+	Asia
A16	+	+	Europe	155	+	+	Asia
A18	−	+	Europe	156	+	+	Asia
A19	−	+	Europe	157	+	+	Asia
A22	+	+	Europe	158	+	+	Asia
A23	−	+	Europe	159	+	+	Asia
A24	+	+	Europe	160	+	+	Asia
A25	+	+	Europe	161	+	+	Asia
A28	+	+	Europe	162	+	+	Asia
A29	+	+	Europe	163	+	+	Asia
A30	+	+	Europe	164	+	−	Asia
A32	+	+	Europe	165	+	+	Asia
A33	+	+	Europe	166	+	+	Asia
A34	+	+	Europe	167	+	+	Asia
A35	+	+	Europe	168	+	+	Asia
A36	+	+	Europe	169	+	+	Asia
A37	+	+	Europe	170	+	+	Asia
A38	+	+	Europe	171	+	+	Asia
A39	+	+	Europe	172	+	+	Asia
A40	+	+	Europe	173	+	+	Asia
A41	+	+	Europe	174	+	+	Asia
A42	+	+	Europe	175	+	+	Asia
A43	+	+	Europe	176	+	+	Asia
A44	+	+	Europe	177	+	+	Asia
A45	+	+	Europe	178	+	+	Asia
A46	−	+	Europe	179	+	+	Asia
A47	+	+	Europe	180	+	+	Asia
A49	+	+	Europe	181	+	+	Asia
A58	−	−	Europe	182	+	+	Asia
A59	+	+	Europe	183	+	+	Asia
A60	−	+	Europe	Ba107	+	+	ND
A61	+	+	Europe	ΔUM-2311	−	−	ND
A62	+	+	Europe	ΔANR-1099	−	−	ND
A63	+	+	Europe	1014	−	+	ND
A64	+	+	Europe	ACB	+	+	ND
A65	+	+	Europe	0074	+	−	ND

[a] Plasmids were detected by PCR (28). +, detected; −, not detected; ND, no data.

TABLE 2. *rpoB Bacillus* species sequences analyzed in this study

Bacillus species	Strain ID	pXO1[a]	pXO2[a]	Derivation or location	Accession no.
B. anthracis	Vollum	−	+	Europe	AF205319
B. anthracis	Ames	+	+	N. America	AF205320
B. anthracis	ΔAmes	−	−	N. America	AF205321
B. anthracis	ΔUM-2311	−	−	Unknown	AF205322
B. anthracis	Sterne	+	−	Africa	AF205323
B. anthracis	Texas 0077	+	−	N. America	AF205324
B. anthracis	001	+	+	Asia	AF205325
B. anthracis	002	+	+	Asia	AF205326
B. anthracis	044	+	−	Asia	AF205327
B. anthracis	A58	−	−	Europe	AF205328
B. anthracis	A74	−	−	Europe	AF205329
B. anthracis	A7	+	+	Europe	AF205330
B. anthracis	AC1	+	−	S. America	AF205331
B. anthracis	7193	+	+	Europe	AF205333
B. anthracis	8403	+	+	Europe	AF205334
B. anthracis	RA3	+	+	Europe	AF205335
B. cereus	14579	−	−	ATCC[b] 14579	AF205336
B. cereus	229	−	−	Unknown	AF205337
B. cereus	27877	−	−	ATCC 27877	AF205338
B. cereus	49069	−	−	ATCC 49069	AF205339
B. cereus	776	−	−	ATCC 19637	AF205341
B. cereus	23261	−	−	ATCC 23261	AF205342
B. mycoides	6462	−	−	ATCC 6462	AF205343
B. thuringiensis	T07-005	−	−	IEBC[c] T07-005	AF205344
B. thuringiensis	T07-146	−	−	IEBC T07-146	AF205345
B. thuringiensis	T07-202	−	−	IEBC T07-202	AF205346
B. thuringiensis	10	−	−	Asia	AF205347
B. thuringiensis	35646	−	−	ATCC 35646	AF205348
B. thuringiensis	B8	−	−	Unknown	AF205349
Bacillus spp.	Ba813_11 (9594/3)	−	−	Europe	AF205350
Bacillus spp.	Ba813_12 (S8553/2)	−	−	Europe	AF205351
Bacillus spp.	Ba813_31 (IB/A)	−	−	Middle East	AF205352
Bacillus sp.	AX16	−	−	Unknown	AF205353
Bacillus sp.	N52	−	−	Unknown	AF205354
Bacillus sp.	V770	−	−	Unknown	AF205355
B. subtilis	6051	−	−	ATCC 6051	AF205356

[a] Ramisse et al., 1996 (28). +, detected; −, not detected; ND, no data.
[b] American Type Culture Collection, Manassas, Va.
[c] International Entomopathogenic *Bacillus* Centre Collection, Pasteur Institute, Paris, France.

bacilli (3). Thus, no absolutely specific chromosomal marker is presently available for the detection of *B. anthracis*.

The *rpoB* gene, which codes for the β-subunit of RNA polymerase, has served as a signature sequence for bacterial identification as well as a locus for phylogenetic analysis (21). Moreover, *rpoB* is a highly conserved housekeeping gene, and at least one copy is present in all bacteria because of its essential role in cellular metabolism. This gene, along with *rpoC*, which encodes for the β′-subunit, constitutes the catalytic center of the pentameric bacterial RNA polymerase (6). Due to its discriminatory power, the *rpoB* gene has been used to develop probes for specific detection and phylogenetic analysis of *Coxiella burnetti*, *Rickettsia*, and *Yersinia pestis* (12, 13, 22).

Bacterial strains and DNA preparation. A total of 144 *B. anthracis* strains from different geographical locations (Table 1), 29 *B. cereus* strains, 49 *B. thuringiensis* strains, 73 *Bacillus* spp. Ba813$^+$ strains (29), a strain each of *B. mycoides*, *B. subtilis*, and *B. megaterium*, and 22 unknown bacilli were used to test the specificity of the assay. Sixteen *B. anthracis* strains and a total of 20 other bacilli strains of *B. cereus* (n = 6), *B. thuringiensis* (n = 6), *B. mycoides* (n = 1), *B. subtilis* (n = 1), and other bacilli (n = 6) were used for the determination of variable region 1 of the *rpoB* gene sequence (Table 2). All strains used in this study were analyzed for plasmid content by a multiplex PCR assay (28). DNA was extracted by a method outlined by Schraft and Griffiths (32) with modifications as described elsewhere (8). For preparing crude vegetative cell lysates, a sterile toothpick was used to transfer a portion of a fresh colony into 300 μl of distilled water. The cell suspension was boiled at 100°C for 15 min and then centrifuged at 8,000 × *g* for 5 min. The supernatant was transferred to a fresh microcentrifuge tube and stored at −20°C until further use. For isolating the rifampin-resistant mutant, an individual fresh colony of the rifampin-sensitive *B. anthracis* 7700 was streaked out on a brain heart infusion agar containing 25 μg of rifampin (Sigma Chemical Co., St. Louis, Mo.) per ml and was incubated overnight at 37°C. Rifampin-resistant colonies were plated out a second time onto a plate containing 50 μg of rifampin/ml in order to confirm this phenotype.

Low-stringency PCR amplification and sequence analysis. The alignment of the amino acid sequences of the RNA polymerase β-subunits of *Bacillus subtilis* and *Escherichia coli* permitted the identification of two conserved regions. The conserved region found near the N terminus was RVIVSQ,

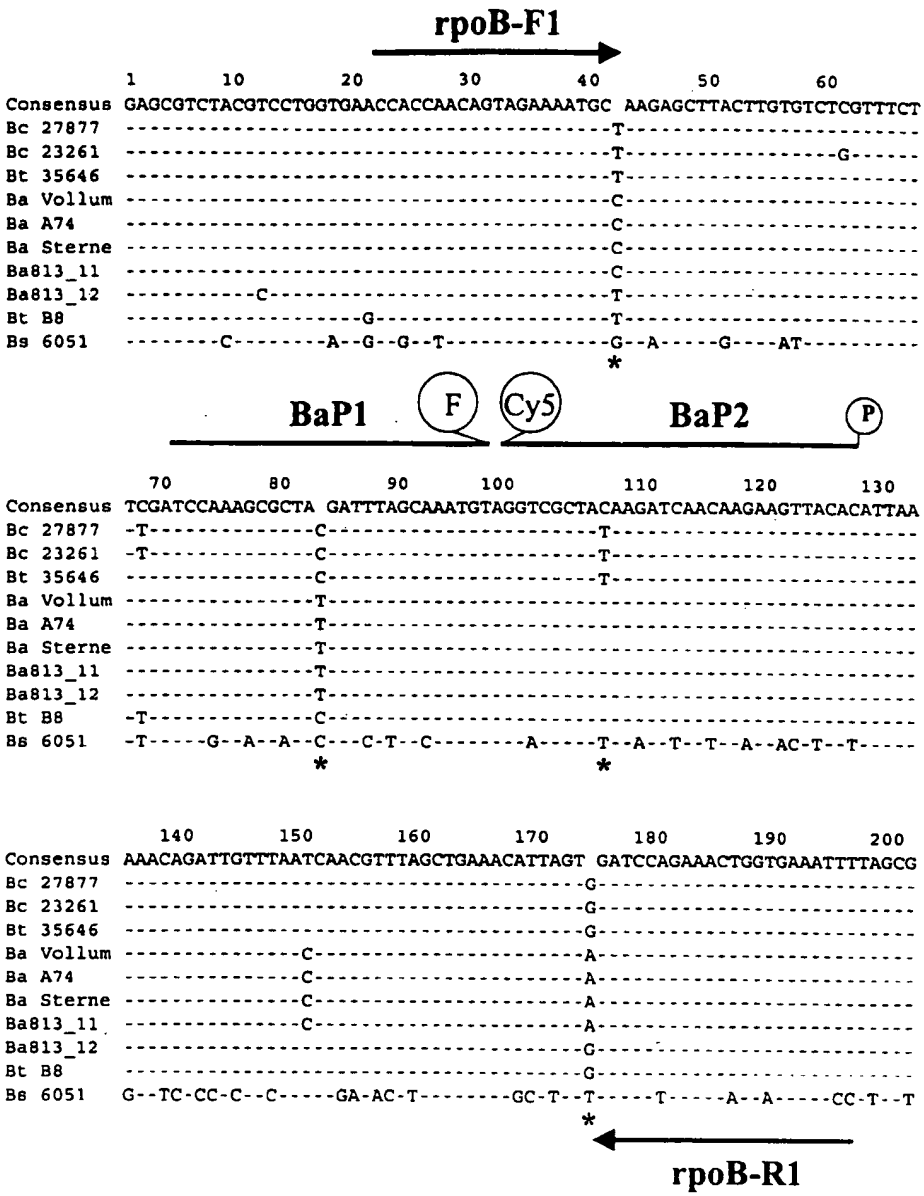

FIG. 1. Alignment of the nucleotide sequences from 10 representative *Bacillus* strains using Clustal W (32). The strains are the following: Bc 27877, *Bacillus cereus* 27877; Bc 23261, *Bacillus cereus* 23261; Bt 35646, *Bacillus thuringiensis* 35646; Ba Vollum, *Bacillus anthracis* Vollum; Ba A74, *Bacillus anthracis* A74; Ba Sterne, *Bacillus anthracis* Sterne; Ba813_11, *Bacillus* sp. strain Ba813_11; Ba813_12, *Bacillus* sp. strain Ba813_12; Bt B8, *Bacillus thuringiensis* BtB8; Bs 6051, *Bacillus subtilis* 6051. The locations of primers and probes are shown (F, fluorescein; Cy5, cyanine 5; P, phosphate group); the presence of an asterisk denotes a mismatch, a dash indicates identity with the consensus sequence, and nucleotide letters indicate positions showing polymorphism.

spanning amino acid residues 132 to 137 of *B. subtilis* and residues 143 to 148 of *E. coli* (6). The C terminus conserved region was DDIDHL, and it was found at positions 399 to 404 of *B. subtilis* and positions 443 to 448 of *E. coli*.

The sequences of primer rpoB1 (5′-CGTGTTATCGTTTC CCAGC-3′) and rpoB2 (5′-AAGATGATCGATATCATC TG-3′) were derived from the two conserved regions and correspond to nucleotides (nt) 1482 to 1500 and 2281 to 2300 of the *B. subtilis rpoB* gene (GenBank accession no. L24376). The PCR reaction mixture of 50 μl consisted of 10 mM Tris-HCl (pH 8.3), 75 mM KCl, 3.5 mM $MgCl_2$, 0.2 mM dNTPs (Boehringer Mannheim Corp., Indianapolis, Ind.), 1 μM (each) primers rpoB1 and rpoB2, 0.05 U of Ampli*Taq* DNA polymerase (Perkin Elmer Corp., Foster City, Calif.)/μl, and 100 ng of DNA template. Amplification was performed in a GeneAmp PCR System 2400 (Perkin-Elmer Corp., Norwalk, Conn.), and the cycling conditions were as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 45°C for 1 min, and 72°C for 1 min, with a final extension of 72°C for 7 min. The amplicons were detected in 2% (wt/vol) SeaKem GTG agarose (FMC Bioproducts, Rockland, Maine) with 40 mM Tris-acetate–1mM EDTA (pH 8.3) as a running buffer and visualized by ethidium bromide staining.

Low-stringency amplification of the variable region of the

TABLE 3. Primers and probes used in the FRET-PCR assay

Primer or probe	Position[a]	T_m (°C)[b]	Sequence (5′→3′)[c]
rpoBF1 primer	(1821–1841)	64.6	CCA CCA ACA GTA GAA AAT GCC
rpoBR1 primer	(1973–1995)	64.2	AAA TTT CAC CAG TTT CTG GAT CT
BaP1 probe	(1871–1897)	74.4	TCC AAA GCG CTA TGA TTT AGC AAA TGT-F
BaP2 probe	(1899–1928)	74.1	Cy5-GGT CGC TAC AAG ATC AAC AAG AAG TTA CAC-P

[a] Based on the *B. subtilis rpoB* gene (GenBank accession no. L24376).
[b] Nearest neighbor method.
[c] F, fluorescein; Cy5, cyanine 5; P, phosphate.

rpoB gene from different *Bacillus* species and strains yielded the expected amplicons with a size of 819 bp. Bands of the expected size were excised from the gel, and the DNA was extracted using a QIAquick Gel Extraction kit (QIAGEN Inc., Valencia, Calif.). The PCR products were cloned into vector pCR 2.1 (Invitrogen Corp., Carlsbad, Calif.) and transformed into *E. coli*. Recombinant plasmids were prepared using the QIAGEN Plasmid Mini Kit. Three clones from each ligation reaction were sequenced in duplicate with the M13 forward and reverse primers using the Applied Biosystems model 373A automated sequencer and the BigDye terminator ready reaction kit (Perkin-Elmer Applied Biosystems). The nucleotide sequences were edited and assembled with the Sequencing Analysis 3.0 and AutoAssembler 3.1.2 programs, respectively; translation into amino acids was accomplished using the Sequence Navigator 3.0.1 program (Perkin-Elmer Applied Biosystems). These 36 sequences were aligned using the Clustal W program (32) from BioNavigator (eBioinformatics Pty Ltd: http://www.ebioinformatics.com/), and four bases specific for *B. anthracis* were identified. Figure 1 shows the alignment and nucleotide differences of 10 representative strains, including 3 strains of *B. anthracis*, 2 strains of *B. cereus*, 2 strains of *B. thuringiensis*, 2 Ba813$^+$ strains of *Bacillus* sp. (29), and a single strain of *B. subtilis*. Thus, nucleotides C (position 42), T (position 84), C (position 108), and A (position 174) are unique to *B. anthracis*, with the exception of Ba813_11 (Fig. 1). The region of the *rpoB* gene described in this study appears to be the only region within the *rpoB* gene that shows variation among different species of bacteria (6).

Nucleotide sequence accession number. The nucleotide sequences of the portion of the *rpoB* gene (variable region 1) described in this study were submitted to GenBank, and the accession numbers are listed in Table 2.

The translation of the nucleotide sequences showed that four bases specific for *B. anthracis* were in the third positions of the codons and did not change the amino acid sequence. The positions of the amino acids in the β-subunit were alanine at 251, tyrosine at 265, tyrosine at 273, and valine at 295. Thus, although there are differences in the nucleotide sequences, no differences were found in the primary sequence of the RpoB proteins for *B. anthracis*, *B. cereus*, and *B. thuringiensis*.

FRET-PCR assay. The primers rpoBF1 and rpoBR1 (Table 3; Fig. 1) were selected for high-stringency PCR amplification using Oligo 6 software (National Biosciences Inc., Plymouth, Minn.). The probes BaP1 (3′ end labeled with Fluorescein) and BaP2 (5′ end labeled with Cy5 and 3′ blocked with a phosphate group) were placed 1 bp apart within the PCR product (Fig. 1) and had T_ms (7) at least 10°C higher than those of the amplification primers (Table 3).

The PCR mixture (10 μl) consisted of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 250 μg of bovine serum albumin/ml (Roche Molecular Biochemicals, Indianapolis, In), 1 μM (each) primers rpoBF1 and rpoBR1, 0.2 μM probe BaP1, 0.4 μM probe BaP2, 0.8U of DNA polymerase Klen*Taq*1 (Ab Peptides, St. Louis, Mo.), and 50 ng of DNA template or 2 μl of crude vegetative cell lysate. The amplification was performed on a Light-Cycler (Idaho Technology, Idaho Falls, Idaho), which is a rapid, forced-air thermocycler with an integrated fluorimeter for real-time monitoring of PCR reactions (35). The amplification was accomplished by initial denaturation at 95°C for 30 s, followed by 35 cycles of 95°C for 0 s, 63°C for 15 s, and 72°C for 5 s. Once the capillaries were placed in the thermocycler, amplification could be completed in less than 30 min. Detection of the amplification products is accomplished by hybridization of a pair of probes to the amplicons as they are formed, resulting in a fluorescence resonance energy transfer (FRET) (35). Fluorescence was measured once every cycle at the annealing step using the F2/F1 filter to monitor amplification in real time. F1 corresponds to the baseline fluorescein fluorescence, while F2 indicates FRET from fluorescein to Cy5, resulting in the ratio of Cy5/fluorescein fluorescence (F2/F1). The increase in fluorescence is proportional to the amount of PCR product generated (7, 35) and is displayed on the computer screen in the real-time mode. The reactions showing an increase in fluorescence by a minimum of 0.05 fluorescence units (y axis) were scored as positive amplification reactions. The PCR products were also visualized by 2% (wt/vol) gel electrophoresis.

The FRET assay was performed on 144 *B. anthracis* strains, harboring any combination of the two plasmids and isolated from different geographical locations (Table 1). All these strains tested positive in the FRET assay, since they displayed an increase in fluorescence as well as the presence of the expected PCR product by agarose gel analysis. Another 175 closely related strains, including the *B. cereus* group and Ba813$^+$ strains, were tested as negative controls to check the specificity of the assay. All related strains, with the exception of Ba813_11, were scored as negative because they did not exhibit an increase in fluorescence. Figure 2 shows the results of the FRET-PCR assay and the electrophoresis of the PCR amplicons using genomic DNA samples from representative strains. *B. cereus*, *B. thuringiensis*, *Bacillus* sp. strain Ba813_12, and *B. subtilis* did not show amplification. The *rpoB* FRET-PCR assay is extremely specific for *B. anthracis* because of the high-stringency PCR conditions coupled with the unique nature of the primers and probes. This specificity occurs at two different levels. The first is at the primer level, as seen in the case of *Bacillus* sp. strain Ba813_12. In this instance PCR products

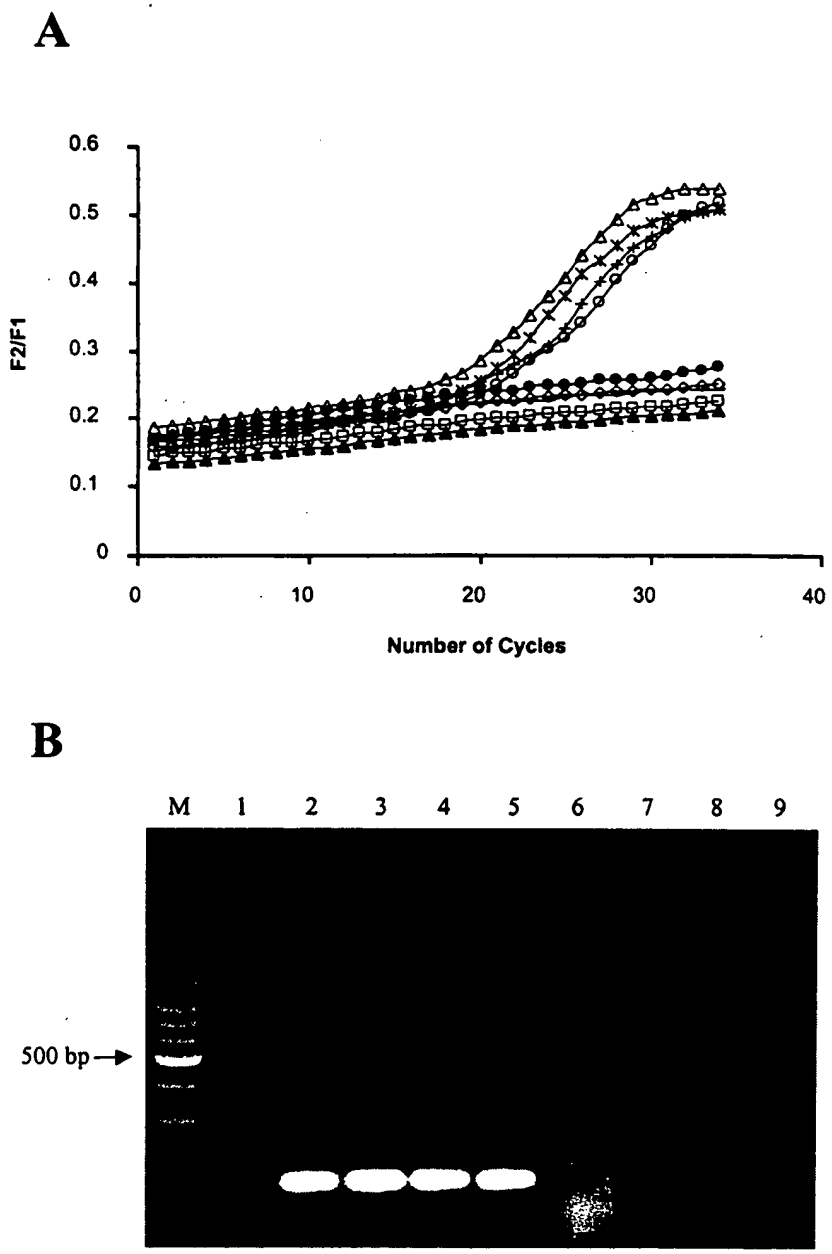

FIG. 2. Results of the FRET-PCR assay using genomic DNA. (A) Fluorescence ratio (F2/F1) is plotted against the number of PCR cycles. The samples are the following: 1, □, negative control (no DNA); 2, △, *Bacillus anthracis* A74; 3, ○, *Bacillus anthracis* Sterne; 4, ✳, *Bacillus* sp. strain Ba813_11; 5, +, *Bacillus anthracis* Vollum; 6, ●, *Bacillus cereus* 27877; 7, ◇, *Bacillus cereus* 23261; 8, −, *Bacillus* sp. strain Ba813_12; 9, ▲, *Bacillus thuringiensis* BtB8. (B) Gel electrophoresis of the PCR products. Lanes, M: 100 bp DNA ladder; 1, negative control (no DNA); samples 2 to 9 are the same as in panel A.

were not generated due to single base-pair difference at 3′ end of both primers, and as a result an increase in fluorescence was not observed in spite of 100% homology of the probe region with the *B. anthracis* sequence. The second level of specificity is at the probe level, since 100% base-pairing of probes with target sequence is required for FRET to occur. A single base-pair mismatch between either of the probe sequences with the target region stops the FRET process, indicating a negative result (unpublished data).

The amplicon derived from *Bacillus* sp. strain Ba813_11 was sequenced, and it was found to have a nucleotide sequence identical to that of *B. anthracis* (Fig. 1). Consequently, the FRET-PCR assay reported here cannot distinguish *Bacillus* sp. strain Ba813_11 from *B. anthracis* strains. The remaining 71 *Bacillus* spp. Ba813$^+$ strains have sequence identical to that of *Bacillus* sp. strain Ba813_12 in primer and probe binding regions, and *Bacillus* sp. strain Ba813_11 appears to be an exception. This strain, *Bacillus* sp. strain Ba813 (9594/3), was

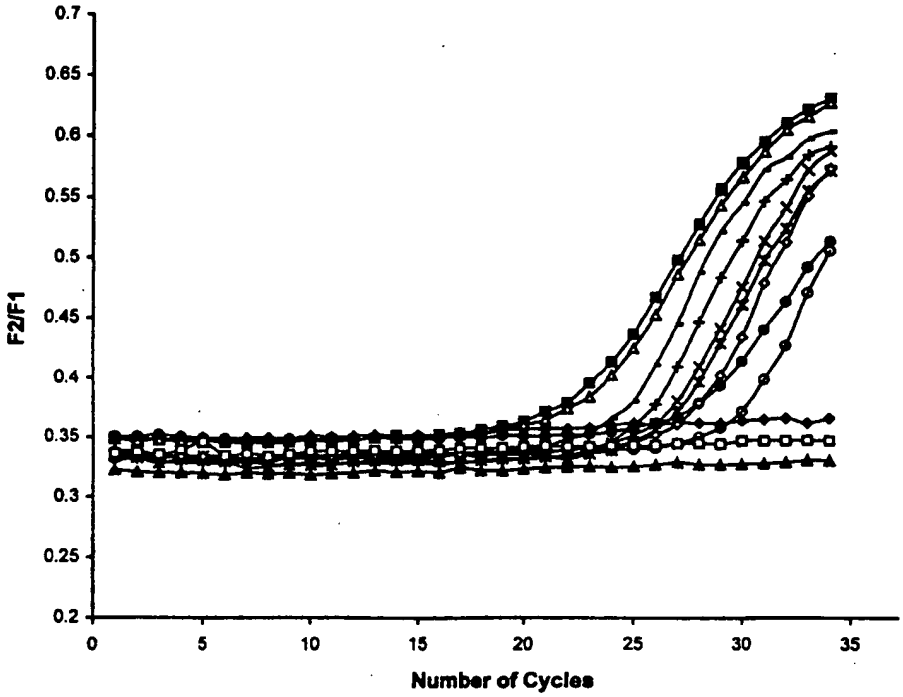

FIG. 3. Results of the FRET-PCR assay using crude vegetative cell lysates. The fluorescence ratio (F2/F1) is plotted against the number of PCR cycles. The sample are the following: ▲, negative control (no DNA); ■, *Bacillus anthracis* AC3; △, *Bacillus anthracis* 7702; ×, *Bacillus anthracis* ΔUM-2311; ✱, *Bacillus anthracis* A74; ●, *Bacillus anthracis* 0074; +, *Bacillus anthracis* Texas 0077; −, *Bacillus anthracis* ΔANR-1099; ○, *Bacillus anthracis* 7700; ◇, *Bacillus anthracis* A58; □, *Bacillus cereus* 14579; ♦, *Bacillus thuringiensis* 10792.

isolated from a station effluent in the Alps in 1997 and was designated a transitional strain because it could not be assigned to a particular species (26, 29). According to the SG-749 locus signature, *Bacillus* sp. strain Ba813_11 belongs to the *B. cereus* group (data not shown) and was shown to contain the Ba813$^+$ marker (26). In contrast to the phenotypic characteristics of *B. anthracis*, the *Bacillus* sp. strain Ba813_11 is hemolytic, motile, and resistant to penicillin, although it has an *rpoB* variable region 1 identical to that of *B. anthracis*.

The assay was not affected by the presence of exogenously added *E. coli* or mixed *Bacillus* species DNA (25 ng of *B. anthracis* DNA + 1,000 ng of exogenously added DNA) representing a mixed microbial community at the ratio of 1:40 (data not shown). The sensitivity of the FRET-PCR assay was examined using different concentrations of exogenously added DNA. Positive fluorescence signals and amplification, as shown by gel electrophoresis, were noticed even when as little as 1 pg of pure genomic DNA was used.

The FRET-PCR assay was also performed on crude vegetative cell lysates from *B. anthracis* and related bacilli. Figure 3 shows the results of the assay on selected strains. Only *B. anthracis* displayed an increase in fluorescence and the presence of the expected amplification product. The increase in fluorescence was observed after 22 to 30 cycles. The magnitude of increase in fluorescence is dependent on the quantity of template DNA or the copy number of the gene target in the reaction (35). It should be noted that the DNA amount was not normalized in the different cell lysates because the number of vegetative cells used in different samples was not identical. Thus, the assay can be directly used on freshly grown cultures for rapid identification of *B. anthracis* strains in less than 1 h. A rifampin-resistant colony exhibited positive results when tested by the FRET-PCR assay because the position of the mutation was found to be outside the *rpoB* target region, as is the case with *B. subtilis* (6). However, if new hotspots are found in the primer and/or probe binding sites, it may not be possible to use the assay for rifampin-resistant *B. anthracis* strains.

The FRET-PCR assay was able to clearly identify and distinguish *B. anthracis* from other closely related bacilli, signifying that the target of this assay is conserved in all strains of *B. anthracis* used in this study and that the detection of *B. anthracis* is independent of the plasmid content. These strains have been isolated from a wide variety of geographic locations, which gives us a reason to believe that this chromosomal marker will continue to be specific to *B. anthracis* even on further investigation. Extensive testing of strains of the *B. cereus* group has shown that the FRET-PCR assay is virtually free of cross-reactivity (99.4% specificity), with the exception of the case of *Bacillus* sp. strain Ba813_11. This assay can be used on endospore suspensions if PCR-amplifiable DNA is released from the spores.

The FRET-PCR assay has several advantages over standard molecular identification techniques. The amplification is monitored in real time, and reactions can be scored as positive or negative without time-consuming routine gel analysis. Moreover, the assay is rapid and highly sensitive when extracted DNA is used as a template for PCR. Using a DNA intercalating fluorescent dye such as SYBR Gold, the specificity of the reaction is evaluated at the end of the PCR amplification (18). The presence of contaminating DNA does not affect the results

of the assay, and hence it can be applied for detection of *B. anthracis* in epidemiological studies and suspected bioterrorist attacks and when analyzing ancient samples. Recent reevaluation of one of the *B. anthracis* strains (Zimbabwe) that was originally determined to be *rpoB* FRET positive has confirmed that it is in fact *rpoB* FRET negative. Moreover, using a newly described technique known as long-range repetitive-element polymorphism–PCR (8), we now have strong evidence suggesting that this strain needs to be regarded as a potential transitional *B. anthracis* strain. We are presently exploring this possibility, and in the meantime we have removed any mention of this particular strain from this report.

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REFERENCES

1. **Andersen, G. L., J. M. Simchock, and K. H. Wilson.** 1996. Identification of a region of genetic variability among *Bacillus anthracis* strains and related species. J. Bacteriol. **178**:377–384.
2. **Anonymous.** 1999. Bioterrorism alleging use of anthrax and interim guidelines for management—United States, 1998. Centers for Disease Control and Prevention. JAMA **281**:787–789.
3. **Ash, C., J. A. Farrow, M. Dorsch, E. Stackebrandt, and M. D. Collins.** 1991. Comparative analysis of *Bacillus anthracis, Bacillus cereus*, and related species on the basis of reverse transcriptase sequencing of 16S rRNA. Int. J. Syst. Bacteriol. **41**:343–346.
4. **Battisti, L., B. D. Green, and C. B. Thorne.** 1985. Mating system for transfer of plasmids among *Bacillus anthracis, Bacillus cereus*, and *Bacillus thuringiensis*. J. Bacteriol. **162**:543–550.
5. **Bernardi, L., G. Vitale, C. Montecucco, and A. Musacchio.** 2000. Expression, crystallization and preliminary X-ray diffraction studies of recombinant *Bacillus anthracis* lethal factor. Acta Crystallogr. D. Biol. Crystallogr. **56**:1449–1451.
6. **Boor, K. J., M. L. Duncan, and C. W. Price.** 1995. Genetic and transcriptional organization of the region encoding the beta subunit of *Bacillus subtilis* RNA polymerase. J. Biol. Chem. **270**:20329–20336.
7. **Breslauer, K. J., R. Frank, H. Blocker, and L. A. Marky.** 1986. Predicting DNA duplex stability from the base sequence. Proc. Natl. Acad. Sci. USA **83**:3746–3750.
8. **Brumlik, M. J., U. Szymajda, D. Zakowska, X. Liang, R. J. Redkar, G. Patra, and V. G. DelVecchio.** 2001. Use of long-range repetitive element polymorphism-PCR to differentiate *Bacillus anthracis* strains. Appl. Environ. Microbiol. **67**:3021–3028.
9. **Cowles, P. B.** 1931. A bacteriophage for *B. anthracis*. J. Bacteriol. **21**:161–166.
10. **Daffonchio, D., S. Borin, G. Frova, R. Gallo, E. Mori, R. Fani, and C. Sorlini.** 1999. A randomly amplified polymorphic DNA marker specific for the *Bacillus cereus* group is diagnostic for *Bacillus anthracis*. Appl. Environ. Microbiol. **65**:1298–1303.
11. **Dixon, T. C., M. Meselson, J. Guillemin, and P. C. Hanna.** 1999. Anthrax. N. Engl. J. Med. **341**:815–826.
12. **Drancourt, M., G. Aboudharam, M. Signoli, O. Dutour, and D. Raoult.** 1998. Detection of 400-year-old *Yersinia pestis* DNA in human dental pulp: an approach to the diagnosis of ancient septicemia. Proc. Natl. Acad. Sci. USA **95**:12637–12640.
13. **Drancourt, M., and D. Raoult.** 1999. Characterization of mutations in the *rpoB* gene in naturally rifampin-resistant Rickettsia species. Antimicrob. Agents Chemother. **43**:2400–2403.
14. **Green, B. D., L. Battisti, T. M. Koehler, C. B. Thorne, and B. E. Ivins.** 1985. Demonstration of a capsule plasmid in *Bacillus anthracis*. Infect. Immun. **49**:291–297.
15. **Harrell, L. J., G. L. Andersen, and K. H. Wilson.** 1995. Genetic variability of *Bacillus anthracis* and related species. J. Clin. Microbiol. **33**:1847–1850.
16. **Helgason, E., O. A. Økstad, D. A. Caugant, H. A. Johansen, A. Fouet, M. Mock, I. Hegna, and A. B. Kolstø.** 2000. *Bacillus anthracis, Bacillus cereus*, and *Bacillus thuringiensis*—one species on the basis of genetic evidence. Appl. Environ. Microbiol. **66**:2627–2630.
17. **Jackson, P. J., E. A. Walthers, A. S. Kalif, K. L. Richmond, D. M. Adair, K. K. Hill, C. R. Kuske, G. L. Andersen, K. H. Wilson, M. Hugh-Jones, and P. Keim.** 1997. Characterization of the variable-number tandem repeats in *vrr*A from different *Bacillus anthracis* isolates. Appl. Environ. Microbiol. **63**:1400–1405.
18. **Lee, M. A., G. Brightwell, D. Leslie, H. Bird, and A. Hamilton.** 1999. Fluorescent detection techniques for real-time multiplex strand specific detection of *Bacillus anthracis* using rapid PCR. J. Appl. Microbiol. **87**:218–223.
19. **Mikesell, P., B. E. Ivins, J. D. Ristroph, and T. M. Dreier.** 1983. Evidence for plasmid-mediated toxin production in *Bacillus anthracis*. Infect. Immun. **39**: 371–376.
20. **Mock, M., E. Labruyere, P. Glaser, A. Danchin, and A. Ullmann.** 1988. Cloning and expression of the calmodulin-sensitive *Bacillus anthracis* adenylate cyclase in *Escherichia coli*. Gene **64**:277–284.
21. **Mollet, C., M. Drancourt, and D. Raoult.** 1997. *rpoB* sequence analysis as a novel basis for bacterial identification. Mol. Microbiol. **26**:1005–1011.
22. **Mollet, C., M. Drancourt, and D. Raoult.** 1998. Determination of *Coxiella burnetii rpoB* sequence and its use for phylogenetic analysis. Gene **207**:97–103.
23. **Okinaka, R., K. Cloud, O. Hampton, A. Hoffmaster, K. Hill, P. Keim, T. Koehler, G. Lamke, S. Kumano, D. Manter, Y. Martinez, D. Ricke, R. Svensson, and P. Jackson.** 1999. Sequence, assembly and analysis of pX01 and pX02. J. Appl. Microbiol. **87**:261–262.
24. **Okinaka, R. T., K. Cloud, O. Hampton, A. R. Hoffmaster, K. K. Hill, P. Keim, T. M. Koehler, G. Lamke, S. Kumano, J. Mahillon, D. Manter, Y. Martinez, D. Ricke, R. Svensson, and P. J. Jackson.** 1999. Sequence and organization of pXO1, the large *Bacillus anthracis* plasmid harboring the anthrax toxin genes. J. Bacteriol. **181**:6509–6515.
25. **Patra, G., P. Sylvestre, V. Ramisse, J. Therasse, and J. L. Guesdon.** 1996. Isolation of a specific chromosomic DNA sequence of *Bacillus anthracis* and its possible use in diagnosis. FEMS Immunol. Med. Microbiol. **15**:223–231.
26. **Patra, G., J. Vaissaire, M. Weber-Levy, C. Le Doujet, and M. Mock.** 1998. Molecular characterization of *Bacillus* strains involved in outbreaks of anthrax in France in 1997. J. Clin. Microbiol. **36**:3412–3414.
27. **Priest, F. G.** 1993. Systematics and ecology of *Bacillus*, p. 3–16. *In* A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and other gram-positive bacteria. American Society for Microbiology, Washington, D.C.
28. **Ramisse, V., G. Patra, H. Garrigue, J. L. Guesdon, and M. Mock.** 1996. Identification and characterization of *Bacillus anthracis* by multiplex PCR analysis of sequences on plasmids pXO1 and pXO2 and chromosomal DNA. FEMS Microbiol. Lett. **145**:9–16.
29. **Ramisse, V., G. Patra, J. Vaissaire, and M. Mock.** 1999. The Ba813 chromosomal DNA sequence effectively traces the whole *Bacillus anthracis* community. J. Appl. Microbiol. **87**:224–228.
30. **Roloff, H., P. Glockner, K. Mistele, and R. Böhm.** 1996. The taxonomic relationship between *B. anthracis* and the *B. cereus* group, investigated by DNA-DNA hybridization and DNA amplification fingerprinting (DAF). Salisbury Med. Bull. **87**(Suppl.):38–39.
31. **Schraft, H., and M. W. Griffiths.** 1995. Specific oligonucleotide primers for detection of lecithinase-positive *Bacillus* spp. by PCR. Appl. Environ. Microbiol. **61**:98–102.
32. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22**:4673–4680.
33. **Turnbull, P. C. B., R. A. Hutson, M. J. Ward, M. N. Jones, C. P. Quinn, N. J. Finnie, C. J. Duggleby, J. M. Krammer, and J. Melling.** 1992. *Bacillus anthracis* but not always anthrax. J. Appl. Microbiol. **72**:21–28.
34. **Uchida, I., T. Sekizaki, K. Hashimoto, and N. Terakado.** 1985. Association of the encapsulation of *Bacillus anthracis* with a 60 megadalton plasmid. J. Gen. Microbiol. **131**:363–367.
35. **Wittwer, C. T., M. G. Herrmann, A. A. Moss, and R. P. Rasmussen.** 1997. Continuous fluorescence monitoring of rapid cycle DNA amplification. Biotechniques **22**:130–8.

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Comparison of primers for the detection of *Salmonella enterica* serovars using real-time PCR

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ABSTRACT

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Aims: To evaluate the specificity and sensitivity of PCR primers for the detection of *Salmonella enterica* in a real-time PCR assay using pure cultures.

Methods and Results: Unenriched whole cells in sterile water were used as template for each PCR. SYBR Green dye was used for the nonspecific detection of dsDNA. The real-time PCR detection limits of five previously published primer sets used in conventional PCR applications were not below 3×10^3 CFU per reaction (rxn). A new primer set, Sen, was designed, which detected *Salm. enterica* Newport down to 6 CFU rxn^{-1} in one case, and gave an average detection limit of 35 CFU rxn^{-1} over three separate runs.

Conclusions: Primers originally designed for end-point PCR did not have adequate specificity or sensitivity compared with those specifically designed for real-time PCR.

Significance and Impact of the Study: This study emphasizes the importance of evaluating real-time PCR primer sets in pure cultures prior to testing in field samples. This study will benefit other researchers in selecting an appropriate primer set for real-time PCR detection of *Salm. enterica*.

Keywords: dissociation analysis, *invA* gene, primer dimer, SYBR Green, threshold cycle.

INTRODUCTION

Real-time PCR offers advantages over traditional PCR in that it is possible to watch the PCR reaction as it occurs, the PCR product does not have to be removed from its reaction chamber for post-PCR analysis, and many detection chemistries are available, including probe-based systems that provide assurance that the correct fragment is amplified. Primer sets that have shown *Salmonella* specificity in traditional PCR applications require evaluation in real-time PCR conditions to determine if these sets are specific and sensitive. Conventional PCR primers that have a strong tendency to form primer dimers may yield poor sensitivity results in a real-time assay. Since primer dimers are dsDNA, they will generate fluorescent signals (Higuchi *et al.* 1992) that yield false-positive results. If the primers form dimers, then at the lower target concentrations it may not be possible to distinguish between target fluorescent signals and primer dimer fluorescent signals by examining plots of relative fluorescence as a function of cycle number. In the absence of conventional PCR primers that yield adequate detection limits in real-time PCR, new primers can be designed or a probe-based system can be designed that yields fluorescence only in the presence of a specific dsDNA target.

Several real-time PCR assays have recently been developed for the detection of *Salmonella* (Hoorfar *et al.* 2000; Eyigor *et al.* 2002; Bhagwat 2004). Previous studies have shown comparisons of primers used in traditional PCR assays (Gooding and Choudary 1999; Malorny *et al.* 2003; Ziemer and Steadham 2003). The purpose of this work was to compare primer sets in a real-time PCR assay and to find a primer set that gives specific and sensitive detection of

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Salmonella enterica serovars associated with food-borne outbreaks from produce.

MATERIALS AND METHODS

Bacterial serovars and growth media

All bacteria used in this study are listed in Table 1. Bacteria were cultured on Luria-Bertani agar plates (LB, Difco/BBL, Sparks, MD, USA). Bacteria from an 18-h culture grown on LB plates were diluted in sterile water to an optical density of 0·2 at 600 nm. Serial dilutions were made in sterile water to obtain appropriate cell densities for PCR testing. Cell densities were quantified by plating 100 μl of the appropriate serial dilution on LB plates. The plates were incubated at 37°C overnight and colonies were counted.

Reagents and primers

Primer set sequences and references are shown in Table 2. All primer sets in Table 2, other than Sen, were shown to be specific for *Salmonella* in previously published research by testing with *Salmonella* and non-*Salmonella* target DNA. SYBR Green Master Mix (Applied Biosystems, Foster City, CA) reagent was used in initial experimentation with the Sal, INVA, ST, Fim, and iroB primer sets at primer concentrations of 500 nmol l^{-1}. However, further experimentation with low *Salm. enterica* cell concentrations indicated that there was a tendency for primer dimer formation. In an attempt to minimize this problem, SYBR Green Core Reagents (Applied Biosystems) were used to allow changes in the reagent concentrations. The final core reagent concentrations used in each 50 μl reaction were: 1X SYBR Green PCR Buffer, 3·0 mmol l^{-1} $MgCl_2$, 1·0 mmol l^{-1} dNTP mix with dUTP, and 1·25 U AmpliTaq Gold. In addition, 0·5 U AmpErase UNG enzyme (Applied Biosystems) was added to all Sen primer set reactions. Reduced concentrations of 250 nmol l^{-1} were tested with the Sal and INVA primer sets in sensitivity testing. All experiments with the Sen primer set were performed at primer concentrations of 250 nmol l^{-1} with the core reagents.

Mixtures of SYBR Green Master Mix or SYBR Green Core Reagents with the appropriate primer set were vortexed prior to transfer to the PCR tubes. The total volume of SYBR Green mix including primers was 30 μl (including water if necessary to adjust the volume to 30 μl). Twenty microlitres of the appropriate *Salm. enterica* serial dilution were then added to each tube to give a total

Table 1 Bacteria used for specificity testing

	Strain	Reference/source
Salm. enterica (serovar, serogroup)		
Saintpaul, B	55	SARB*
Schwarzengrund, B	96E01152C-TX-1	Inami and Moler (1999); CDHS†
Mbandaka, C_1	99A1670	CDHS
Newport, C_2	96E01153C-TX	Inami and Moler (1999); CDHS
Albany, C_3	96E01152C-TH	CDHS
Enteritidis, D_1	00A-2768	CDHS
Baildon, D_2	99A-23	CDHS
Meleagridis, E_1	96A7406	CDHS
Senftenberg, E_4	59	SARB
Rubislaw, F	54	SARB
Poona, G_1	00A3563	Barak *et al.* (2003); CDHS
Cubana, G_2	98A9878	Mohle-Boetani *et al.* (2001); CDHS
Havana, G_2	98A4399	Mohle-Boetani *et al.* (2001); CDHS
Saphra, I	97A3312	Mohle-Boetani *et al.* (1999); CDHS
Non-*Salmonella* species		
E. coli O137:H41	MW421	Wachtel *et al.* (2002)
Rahnella aquatilis	SPS2F10	Barak *et al.* (2002)
Pseudomonas putida	BM19	Barak *et al.* (2002)
Pantoea agglomerans	SPS2F1	Barak *et al.* (2002)
Erwinia chrysanthemi	3937	N. Perna‡
Klebsiella pneumonia	S48565.3	SFGH§

*SARB, *Salmonella* reference collection B.
†CDHS, California Department of Health and Human Services.
‡Nicole Perna, University of Wisconsin-Madison.
§SFGH, San Francisco General Hospital.

Table 2 Primer target, name, sequence, expected amplicon length and reference

Target fragment	Primer sets	Primer (5′ → 3′)	Product size (bp)	Reference
invA gene	Sal-3	TAT CGC CAC GTT CGG GCA A	275	Wang *et al.* (1997)
	Sal-4	TCG CAC CGT CAA AGG AAC C		
invA gene	INVA-1	ACA GTG CTC GTT TAC GAC CTG AAT	244	Chiu and Ou (1996)
	INVA-2	AGA CGA CTG GTA CTG ATC GAT AAT		
iroB gene	Primer 1	TGC GTA TTC TGT TTG TCG GTC C	606	Bäumler *et al.* (1997)
	Primer 2	TAC GTT CCC ACC ATT CTT CCC		
Random fragment	ST11	GCC AAC CAT TGC TAA ATT GGC GCA	429	Soumet *et al.* (1999)
of *Salm.* Typhimurium	ST15	GGT AGA AAT TCC CAG CGG GTA CTG G		
fimA gene	Fim1A	CCT TTC TCC ATC GTC CTG AA	85	Cohen *et al.* (1996)
	Fim2A	TGG TGT TAT CTG CCC GAC CA		
invA gene	Sen-1	TTT CAA TGG GAA CTC TGC	172	This work
	Sen-2	AAC GAC GAC CCT TCT TTT		

reaction volume of 50 μl. Negative control reactions used 20 μl of sterile water to make a total reaction volume of 50 μl.

Polymerase chain reactions were performed with a GeneAmp 5700 Sequence Detection System (Applied Biosystems) using the following thermal cycling conditions: 94°C for 5 min; 40 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, with a final extension of 72°C for 7 min. The annealing temperature was lowered to 54°C for the Sen primer set due to its lower melting temperature. Prior to the 94°C hold for 5 min, an initial step of 50°C for 2 min was added to the Sen primer set protocol to activate the AmpErase UNG enzyme. After each PCR, a dissociation profile was generated by increasing the temperature from 60 to 90°C in increments of 0·4°C over 20 min, while measuring changes in fluorescence.

Gel analysis

In addition to dissociation curve analysis, PCR amplification fragments were run in 2% agarose gels in Tris–acetate–EDTA buffer to confirm fragment size. Following the PCR, 2 μl of 6X tracking dye (Promega, Madison, WI, USA) were mixed into the 50 μl reaction volume and 10 μl of this mixture were used for gel electrophoresis. A Hi-Lo DNA ladder (Minnesota Molecular, Minneapolis, MN, USA) was used to compare fragment sizes. Gels were stained with SYBR Green I (Molecular Probes, Eugene, OR, USA) or ethidium bromide for 10–20 min. The agarose gels were then photographed under u.v. excitation.

Primer design

Software (Primer Premier 5.0, PREMIER Biosoft International, Palo Alto, CA, USA) was used for assessment of the previously published primer sets and design of a new one. Given a particular target sequence and primer set, the software calculates the Gibbs free energy (ΔG) required for specific and nonspecific products to form, thereby allowing primers to be evaluated prior to experimentation. A large negative ΔG means that a reaction is energetically favourable and, therefore, indicates a tendency to form a dsDNA product. In addition to searching for potential primer dimers, the software can find locations outside of the target region where primers have a tendency to anneal, leading to nonspecific product formation called false priming. The *invA* gene from *Salm. enterica* serovar Typhimurium LT2 (accession number: AE008832, McClelland *et al.* 2001) was used as target DNA for software analysis of the Sal and INVA primer sets, and for designing a new primer set.

DNA sequencing

PCR products from the Sen primer set were sequenced to make certain that the target sequence was amplified. PCR products for sequencing were produced with the Sen primer set real-time PCR procedure described previously. DNA samples were purified with a QIAquick gel extraction kit (Qiagen, Valencia, CA, USA). Sequencing was performed by the Division of Biological Sciences DNA Sequencing Facility (University of California, Davis, CA, USA).

Experimental procedures

Each primer set was evaluated for its ability to amplify products from *Salm. enterica* serovars and from low initial template concentrations (sensitivity). Experimental conditions and procedures are summarized in Table 3. The Sal, INVA, iroB, ST, and Fim primer sets were tested in one experiment with the *Salm. enterica* serovars listed in Table 1 at 10^3CFU per reaction (rxn), and the Sen primer set was tested in one experiment with all bacteria shown in Table 1 at 10^3 CFU rxn^{-1}. Dissociation plot peaks and amplification fragments on agarose gels were used to verify that the

Table 3 Procedures and experimental conditions

Procedure	Primer set	[Primer] (nmol l^{-1})	PCR reagents*	Bacteria	[Bacteria] (CFU rxn^{-1})
Initial screening, traditional primers	Sal, INVA, iroB, Fim, ST	500	Master Mix	*Salm. enterica* serovars, Table 1	10^3
Sensitivity	Sal, INVA	250	Core Reagents	*Salm. enterica* Newport	10^6–10^3
Software analysis	Sal, INVA, Sen	–	–	–	–
Specificity	Sen	250	Core Reagents	All bacteria, Table 1	10^3
Sensitivity	Sen	250	Core Reagents	*Salm. enterica* Newport	10^6–10^0

*SYBR Green Master Mix or Core Reagents.

correct sequences were amplified. A primer set was considered for sensitivity testing if the initial results indicated single peaks in the dissociation plots, no peaks in the negative control dissociation plots and if agarose gel electrophoresis indicated that the correct size fragment was formed. Sensitivity testing involved three replicates per experiment using three separate experiments with each primer set. *Salm. enterica* Newport, an outbreak strain associated with produce, was used for sensitivity testing with levels ranging from 10^6 to 10^3 CFU rxn^{-1} for the Sal and INVA primer sets and approximately 10^6 to 10^0 CFU rxn^{-1} for the Sen primer set.

The detection limit of a particular primer set was determined by comparing the mean threshold cycle (C_T) value of the dilution with the mean C_T value of the negative control reactions. In the case of the Sal and INVA primer sets a one-tailed Student's *t*-test with $\alpha = 0{\cdot}05$ was performed to determine if there was a significant difference between the mean C_T values of the negative control reactions and the reactions containing the *Salm. enterica* Newport dilution of interest. Primer set detection limits were established by determining the lowest *Salm. enterica* Newport cell density mean C_T that was significantly different from the negative control.

RESULTS

Testing Sal, INVA, iroB, Fim, and ST primer sets using *Salm. enterica* serovars

The Sal, INVA, iroB, Fim and ST primer sets were tested with all *Salm. enterica* serovars shown in Table 1. Figure 1(a,b) show typical dissociation plots with each of the primer sets. The dissociation plots of the iroB and ST primer sets displayed multiple peaks with each serovar, while agarose gel electrophoresis showed a single band at the expected size in all cases. Without sequencing the PCR products, it was difficult to conclude if single or multiple products were formed with the iroB and ST primer sets. Furthermore, the iroB gel results produced faint bands and small dissociation peaks for *Salm. enterica* serovars Newport, Albany, Enteritidis and Baildon with significant amounts of primer dimer formation seen in these reactions. Thus, the iroB and ST primer sets were not used in the sensitivity experiments.

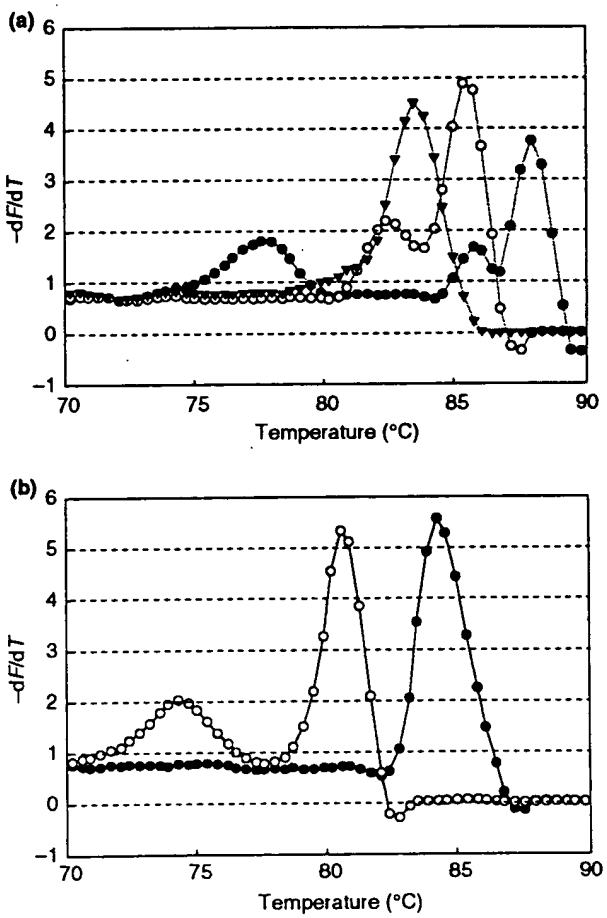

Fig. 1 Results of specificity testing using the iroB, ST, Fim, Sal, and INVA primer sets with *Salm. enterica* Schwarzengrund at a concentration of 6×10^4 CFU rxn^{-1}. Primer sets: (a) iroB (●), ST (○), Fim (▼); (b) Sal (●), INVA (○)

The Fim primer set produced a single 85 bp product and a single peak in the dissociation plot. However, primer dimers formed in the negative control reaction of the same size and position on the dissociation plot as the expected product. Testing this primer set at a reduced concentration of 250 nmol l^{-1} could help to reduce primer dimer formation. However, even if the size of the primer dimer seen in the negative control reaction was reduced, it may be difficult to interpret results from the *Salm. enterica* reactions because it will not be possible to tell if the fluorescent signal generated is from the target product, a primer dimer, or a combination of both. As it was not possible to distinguish between the *Salm. enterica* reactions and the negative control reactions in either dissociation analysis or gel electrophoresis, the Fim primer set was not used for further experimentation.

The INVA primer set results indicated that products of the correct size were formed with all *Salm. enterica* serovars tested. Figure 1(b) shows a large primary peak at 80°C along with a smaller secondary peak (which may have been a primer dimer) at a melting temperature of about 74°C. The larger peak in the dissociation analysis corresponded to the 244 bp product seen in the agarose gel (data not shown), as expected. As the primary product peak was clearly distinguishable from the smaller peak, the INVA primer set was used for sensitivity testing.

All *Salm. enterica* serovars tested with Sal were detected at 10^3 CFU rxn^{-1} with little to no primer dimer formation. The peak seen at 85°C in the dissociation plot of Fig. 1(b) corresponded to the expected 275 bp product seen in the gel analysis. The Sal primer set yielded the expected product for all *Salm. enterica* serovars and was therefore selected for sensitivity analysis.

Sensitivity of the Sal and INVA primer sets

In the case of both Sal and INVA primer sets, the dissociation plots showed that as the amount of initial template decreased, the height of the primer dimer peak increased and the height of the product peak decreased (data not shown). The detection limits of the Sal and INVA primer sets were approx. 3×10^3 and 3×10^4 CFU rxn^{-1}, respectively.

Primer analysis and design

As a lower detection limit was desired, software was used to compare the Sal and INVA primer sets with a new primer set design. Analysis of INVA and Sal indicated that these primer sets might form primer dimers, as indicated by the relatively large negative ΔG values associated with different primer dimer formations (data not shown).

A new primer set, Sen (*Salm. enterica*), also targeting the *invA* gene was designed and tested for *Salm. enterica* specificity using *Salm. enterica* and non-*Salmonella* isolates. The detection limit of Sen was also tested with *Salm. enterica* Newport. Table 2 gives the Sen primer set sequence. The software results indicated that only the reverse primer, Sen-2, has the potential for false priming on the *invA* gene (data not shown).

Sen primer set specificity and sensitivity

All *Salmonella* serovars shown in Table 1 formed a single product near the expected size, based on the real-time PCR dissociation plots and agarose gel electrophoresis results (data not shown). Sen primer set PCR products were sequenced in both directions and the product formed was the expected length of 172 bp. A BLAST analysis of the sequence results indicated that the correct sequence of bases was formed (accession number: AE008832, McClelland *et al.* 2001). None of the non-*Salmonella* bacteria crossed the real-time PCR threshold value, Rn = 0·5, over the course of 40 cycles (data not shown). Therefore, this primer set was considered specific for *Salm. enterica*.

Typical sensitivity results with the Sen primer set are shown in Fig. 2. Figure 2(a) shows that *Salm. enterica* Newport was detectable from 6×10^5 to 6×10^0 CFU rxn^{-1}, while the negative control reaction did not generate visible dsDNA. Additionally, as seen in Fig. 2b, no primer dimers were formed with the *Salm. enterica* Newport dilutions and all product peaks were found at a temperature of 82 ± 1°C.

The Sen sensitivity results indicate that the negative control reactions did not generate enough dsDNA to cross the relative fluorescence threshold value. As no negative control C_T values were available for comparison with *Salm. enterica* reaction C_T values, no statistical tests were performed. Detection limits of 80, 6 and 20 CFU rxn^{-1} were found in three separate runs, giving an average detection limit of 35 CFU rxn^{-1} with a standard deviation of 39 CFU rxn^{-1}.

DISCUSSION

In comparing the six primer sets for detection of *Salm. enterica* with real-time PCR, we chose to use pure cultures in sterile water as the first step in assay development. The formation of primer dimers in real-time PCR applications generates false-positive signals (Higuchi *et al.* 1992). These primer dimers ultimately lead to poor detection limits even without the presence of inhibiting substances found in field samples.

With varying degrees of amplification, all primer sets tested with the *Salm. enterica* serovars shown in Table 1 formed PCR products of the expected sizes at

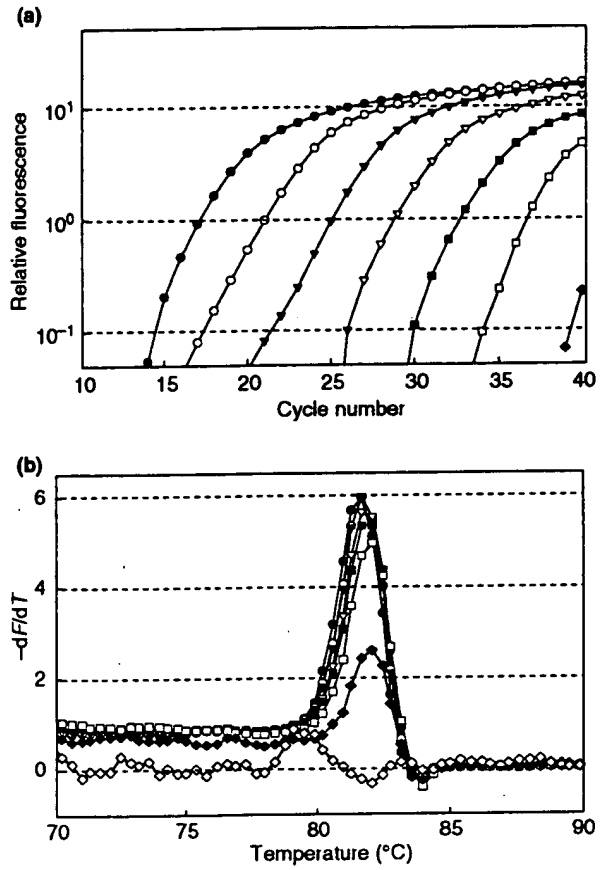

Fig. 2 Amplification and dissociation plots with the Sen primer set amplifying the DNA of *Salm. enterica* Newport. Cell concentrations: (a) 6×10^5 CFU rxn^{-1} (●), 6×10^4 CFU rxn^{-1} (○), 6×10^3 CFU rxn^{-1} (▼), 6×10^2 CFU rxn^{-1} (▽), 6×10^1 CFU rxn^{-1} (■), 6×10^0 CFU rxn^{-1} (□), 6×10^{-1} CFU rxn^{-1} (◆). Cell concentrations: (b) 6×10^5 CFU rxn^{-1} (●), 6×10^4 CFU rxn^{-1} (○), 6×10^3 CFU rxn^{-1} (▼), 6×10^2 CFU rxn^{-1} (▽), 6×10^1 CFU rxn^{-1} (■), 6×10^0 CFU rxn^{-1} (□), 6×10^{-1} CFU rxn^{-1} (◆), negative control (◇)

10^3 CFU rxn^{-1}. However, multiple peaks were seen in the dissociation plots of the ST and iroB primer sets with most of the *Salm. enterica* serovars tested, and this may have been the result of two products formed that were close to one another or a single product with a DNA sequence that generates a complex melting profile. Li *et al.* (2003) demonstrated that complex melting profiles can result from small single PCR products with SYBR Green dye. Their data indicate that regions of high A/T and G/C bases contribute to the shape of the melting profile of a product. A complex melting profile in the dissociation analysis of a PCR product that shows a single band on a gel requires sequencing to confirm that one product was actually formed and co-migrating bands were not present.

With the exception of one sample at 8 CFU rxn^{-1}, all dissociation plots for Sen primer products displayed single peaks larger than the background signal. The dissociation analyses confirmed that the correct melting temperature was found in each of the Sen primer set products generated.

Although extraction and enrichment procedures may improve the sensitivity of PCR, both steps are time consuming and labour intensive and, thus, were not used here to prepare the samples. Without an enrichment technique to increase levels of target DNA or an extraction process to purify DNA from a sample, the choice of primer set is critical in a real-time PCR application. At high template concentrations (10^6–10^5 CFU rxn^{-1}), both the Sal and INVA primer sets were consistently able to amplify the target fragment of interest. However, PCR with less template often generated primer dimers, ultimately yielding poor detection limits. The primer software was valuable in predicting the tendency for dimer formation and designing the Sen primer set which was ultimately the most sensitive primer set we tested. Using primers developed for traditional PCR may lower real-time assay sensitivity. Therefore, new primers are required specifically for real-time PCR.

The ultimate goal of this research is to build an automated biosensor for field use to screen for pathogens in the irrigation/wash water from fruits and vegetables. Jackson *et al.* (2003) started this work and developed components for such a biosensor. An ideal biosensor rapidly confirms the presence or absence of its target. While enrichment techniques have been used prior to PCR for sensitive detection of *Salmonella* (Eyigor *et al.* 2002; Bhagwat 2004), these procedures typically take hours and such practices are incompatible with the goal of rapid detection. In order to improve our detection limit, an automated concentration mechanism is under development for the purpose of increasing the cell densities of liquid test samples. DNA extraction steps may also improve PCR target detection, but not all steps in an extraction process are easily automated. Obtaining a specific and sensitive primer set for real-time PCR detection of *Salm. enterica* under ideal conditions was the first step in the development of an automated biosensor. The next step will be testing *Salm. enterica* detection performance in spiked water samples from produce operations.

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REFERENCES

Barak, J.D., Whitehand, L.C. and Charkowski, A.O. (2002) Differences in attachment of *Salm. enterica* serovars and *Escherichia coli* O157:H7 to alfalfa sprouts. *Applied and Environmental Microbiology* 68, 4758–4763.

Barak, J.D., Chue, B. and Mills, D. (2003) Recovery of surface bacteria from and surface sanitization of cantaloupes. *Journal of Food Protection* 66, 1805–1810.

Bäumler, A.J., Heffron, F. and Reissbrodt, R. (1997) Rapid detection of *Salm. enterica* with primers specific for *iroB*. *Journal of Clinical Microbiology* 35, 1224–1230.

Bhagwat, A.A. (2004) Rapid detection of *Salmonella* from vegetable rinse-water using real-time PCR. *Food Microbiology*, 21, 73–78.

Chiu, H.-C. and Ou, J.T. (1996) Rapid identification of *Salmonella* serovars in feces by specific detection of virulence genes, *invA* and *spvC*, by an enrichment broth culture-multiplex PCR combination assay. *Journal of Clinical Microbiology* 34, 2619–2622.

Cohen, H.J., Mechanda, S.M. and Lin, W. (1996) PCR amplification of the *fimA* gene sequence of *Salmonella* Typhimurium, a specific method for detection of *Salmonella* spp. *Applied and Environmental Microbiology* 62, 4303–4308.

Eyigor, A., Carli, K.T. and Unal, C.B. (2002) Implementation of real-time PCR to tetrathionate broth enrichment step of *Salmonella* detection in poultry. *Letters in Applied Microbiology* 34, 37–41.

Gooding, C.M. and Choudary, P.V. (1999) Comparison of different primers for rapid detection of *Salmonella* using the polymerase chain reaction. *Molecular and Cellular Probes* 13, 341–347.

Higuchi, R., Dollinger, G., Walsh, S.P. and Griffith, R. (1992) Simultaneous amplification and detection of specific DNA sequences. *BioTechnology* 10, 413–417.

Hoorfar, J. Ahrens, P. and Rådström, P. (2000) Automated 5′ nuclease PCR assay for identification of *Salmonella enterica*. *Journal of Clinical Microbiology* 38, 3429–3435.

Inami, G.B. and Moler, S.E. (1999) Detection and isolation of *Salmonella* from naturally contaminated alfalfa seeds following an outbreak investigation. *Journal of Food Protection* 62, 662–664.

Jackson, E., Delwiche, M., Barak, J., Charkowski, A. and Suslow, T. (2003) Sensor components for PCR detection of *Salmonella* in alfalfa sprout irrigation water. *Transactions of the ASAE* (in press).

Li, W., Xi, B., Yang, W., Hawkins, M. and Schubart, U.K. (2003) Complex DNA melting profiles of small PCR products revealed using SYBR Green I. *BioTechniques* 35, 702–706.

Malorny, B., Hoofar, J., Bunge, C. and Helmuth, R. (2003) Multicenter validation of the analytical accuracy of *Salmonella* PCR: towards an international standard. *Applied and Environmental Microbiology* 69, 290–296.

McClelland, M., Sanderson, K.E., Spieth, J., Clifton, S.W., Latreille, P., Courtney, L., Porwollik, S., Ali, J. *et al.* (2001) Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. *Nature* 413, 852–856.

Mohle-Boetani, J.C., Reporter, R., Werner, S.B., Abbott, S., Farrar, J., Waterman, S.H. and Vugia, D.J. (1999) An outbreak of *Salmonella* serogroup Saphra due to cantaloupes from Mexico. *Journal of Infectious Diseases* 180, 1361–1364.

Mohle-Boetani, J.C., Farrar, J.A., Werner, S.B., Minassian, D., Bryant, R., Abbott, S., Slutsker, L. and Vugia, D.J. (2001) *Escherichia coli* O157 and *Salmonella* infections associated with sprouts in California, 1996–1998. *Annals of Internal Medicine* 135, 239–247.

Soumet, C., Ermel, G., Rose, N., Rose, V., Drouin, P., Salvat, G. and Colin, P. (1999) Evaluation of a multiplex PCR assay for simultaneous identification of *Salmonella* sp., *Salmonella* Enteritidis and *Salmonella* Typhimurium from environmental swabs of poultry houses. *Letters in Applied Microbiology* 28, 113–117.

Wachtel, M.R., Whitehand, L.C. and Mandrell, R.E. (2002) Prevalence of *Escherichia coli* associated with a cabbage crop inadvertently irrigated with partially treated sewage wastewater. *Journal of Food Protection* 65, 471–475.

Wang, R.-F., Cao, W.-W. and Cerniglia, C.E. (1997) A universal protocol for PCR detection of 13 species of foodborne pathogens in foods. *Journal of Applied Microbiology* 83, 727–736.

Ziemer, C.J. and Steadham, S.R. (2003) Evaluation of the specificity of *Salmonella* PCR primers using various intestinal bacterial species. *Letters in Applied Microbiology* 37, 463–469.

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Multiplex PCR: Optimization and Application in Diagnostic Virology

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INTRODUCTION

During the past decade, advances in PCR technology and other DNA signal and target amplification techniques have resulted in these molecular diagnostics becoming key procedures (4, 107, 117). Such techniques are conceptually simple, highly specific, sensitive, and amenable to full automation (54, 115). The most mature of these technologies, PCR, is in one variant or another now common in research laboratories and is used increasingly in routine diagnostic laboratory settings and undergraduate and high-school teaching (32, 38, 40, 101). In diagnostic laboratories the use of PCR is limited by cost and sometimes the availability of adequate test sample volume. To overcome these shortcomings and also to increase the diagnostic capacity of PCR, a variant termed multiplex PCR has been described. In multiplex PCR more than one target sequence can be amplified by including more than one pair of primers in the reaction. Multiplex PCR has the potential to produce considerable savings of time and effort within the laboratory without compromising test utility. Since its introduction, multiplex PCR has been successfully applied in many areas of nucleic acid diagnostics, including gene deletion analysis (19, 20), mutation and polymorphism analysis (86, 96), quantitative analysis (94, 124), and RNA detection (51, 126). In the field of infectious diseases, the technique has been shown to be a valuable method for identification of viruses, bacteria, fungi, and/or parasites. A representative list of such agents is shown in Table 1.

Based upon our own experience with multiplex PCR and those of other authors appearing in the literature during the last 10 years, we review the theoretical and practical basis of the development and optimization of multiplex PCR systems and discuss the application and potential of this technique in the field of diagnostic virology.

PRINCIPLE AND DEVELOPMENT OF MULTIPLEX PCR

A number of review and research articles have provided detailed descriptions of the key parameters that may influence the performance of standard (uniplex) PCR (17, 57, 88, 91, 112). Fewer publications discuss multiplex PCR (18, 28, 43).

Primers and Multiplex PCR Efficiency

The first few rounds of thermal cycling have substantial effect on the overall sensitivity and specificity of PCR (92). Assuming efficient denaturation of the target, overall success of specific amplification depends on the rate at which primers anneal to their target and the rate at which annealed primers are extended along the desired sequence during the early, middle, and late cycles of the amplification. Factors preventing optimal annealing rates include poorly designed primers and suboptimal buffer constituents and annealing temperature. The extension rate of specific primer-target hybrids depends on the activity of the enzyme, availability of essential components such as deoxyribonucleoside triphosphates (dNTPs), and the nature of the target DNA. Thus, the majority of modifications to improve PCR performance have been directed towards the factors affecting annealing and/or extension rates.

The optimization of multiplex PCRs can pose several difficulties, including poor sensitivity or specificity and/or preferential amplification of certain specific targets (76). The presence of more than one primer pair in the multiplex PCR increases the chance of obtaining spurious amplification products, primarily because of the formation of primer dimers (9). These nonspecific products may be amplified more efficiently than the desired target, consuming reaction components and producing impaired rates of annealing and extension. Thus, the optimization of multiplex PCR should aim to minimize or

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TABLE 1. Representative list of applications of multiplex PCR to the diagnosis of infectious diseases

Infectious agent	Pathogens targeted	Clinical manifestation(s) and/or specimen	Reference(s)
Virus	HIV-1, HIV-2, HTLV-1, and HTLV-2	Blood	45
	HSV-1, HSV-2, VZV, CMV, HHV-6, EBV, and EVs[a]	Meningitis, encephalitis, or meningoencephalitis; CSF	13, 14
Bacterium	*Haemophilus influenzae*, *Streptococcus pneumonia*, *Mycoplasma catarrhalis*, and *Alloiococcus otitidis*	Upper respiratory tract	42
	Campylobacter jejuni and *Campylobacter coli*	Human campylobacteriosis	37
	Actinomyces actinomycetemcomitans, *Porphyromonas intermedia*, and *Porphyromonas gingivalis*	Periodontal infection	34
	N. gonorrhoeae and *C. trachomatis*	Genital infections	60, 118
	C. trachomatis, *N. gonorrhoeae*, *Ureaplasma urealyticum*, and *M. genitalium*	Genital infections	59
Parasite	*Giardia lamblia* and *Cryptosporidium parvum*	Diarrheal disease; water	52, 89
	Leishmania spp.	Leishmaniasis	5, 39
Combination	HSV, *H. ducreyi*, and *T. pallidum*	Genital ulcer disease	7, 73
	HPVs, HSV, and *C. trachomatis*	Genital swabs	64
	Adenovirus, HSV, and *C. trachomatis*	Keratoconjunctivitis	Yeo et al.[b]
	EV, influenza viruses A and B, RSV, PIV types 1 and 3, adenovirus, *M. pneumoniae*, and *C. pneumoniae*	Acute respiratory tract infections	36

[a] EVs, enteroviruses.
[b] Abstr. 97th Gen. Meet. Am. Soc. Microbiol., 1997.

reduce such nonspecific interactions. Empirical testing and a trial-and-error approach may have to be used when testing several primer pairs, because there are no means to predict the performance characteristics of a selected primer pair even among those that satisfy the general parameters of primer design (43). However, special attention to primer design parameters such as homology of primers with their target nucleic acid sequences, their length, the GC content, and their concentration have to be considered (26, 65, 72, 88, 95, 120). Ideally, all the primer pairs in a multiplex PCR should enable similar amplification efficiencies for their respective target. This may be achieved through the utilization of primers with nearly identical optimum annealing temperatures (primer length of 18 to 30 bp or more and a GC content of 35 to 60% may prove satisfactory) and should not display significant homology either internally or to one another (17, 26, 43).

Preferential amplification of one target sequence over another (bias in template-to-product ratios) is a known phenomenon in multiplex PCRs that are designed to amplify more than one target simultaneously (68, 76, 109). Based on both theoretical modeling and experimental studies, two major classes of processes that induce this bias have been identified, PCR drift and PCR selection (108). PCR drift is a bias assumed to be due to stochastic fluctuation in the interactions of PCR reagents particularly in the early cycles, which could arise in the presence of very low template concentrations (26, 68); variations in the thermal profiles of a thermocycler, resulting in unequal ramping temperatures; or simple experimental error. PCR selection, on the other hand, is defined as a mechanism which inherently favors the amplification of certain templates due to the properties of the target, the target's flanking sequences, or the entire target genome. These properties include interregion differences in GC content, leading to preferential denaturation; higher binding efficiency because of GC-rich primers; differential accessibility of targets within genomes due to secondary structures; and the gene copy number within a genome. In addition, the choice of primers has been shown to be crucial to avoid PCR selection. Amplification biases that were strongly dependent on the choice of primers and dependent to a lesser extent on the templates have been described (100). Some primer pairs with high amplification efficiency resulted in templates being saturated (plateau phase), while other primer pairs produced product independent of starting template concentrations. Primers with lower amplification efficiency resulted in product concentrations below the saturation concentrations, and depending on the template, either the expected product ratio or bias was observed.

Other PCR Components

Alteration of other PCR components such as PCR buffer constituents, dNTPs, and enzyme concentrations in multiplex PCR over those reported for most uniplex PCRs usually results in little, if any, improvement in the sensitivity or specificity of the test. Increasing the concentration of these factors may increase the likelihood of mis-priming with subsequent production of spurious nonspecific amplification products. However, optimization of these components in multiplex PCRs that are designed for simultaneous amplification of multiple targets may prove beneficial. For example, in the multiplex PCR for the dystrophin gene (nine genomic targets), a *Taq* DNA polymerase concentration (with an appropriate increase in $MgCl_2$ concentration) four to five times greater than that required in uniplex PCR was necessary to achieve optimal nucleic acid amplification (19). Variation in concentrations of reaction components above those used in uniplex PCR probably reflects the competitive nature of the PCR process. The desired target DNA can be outcompeted by the more efficient amplification of other targets (including nonspecific products), leading to decreases in the efficiency of the amplification of the desired targets and hence sensitivity of the reaction (79).

PCR additives, such as dimethyl sulfoxide, glycerol, bovine serum albumin, or betaine, have been reported to be of benefit in multiplex PCRs (49, 62). The components may act to prevent the stalling of DNA polymerization, which can occur through the formation of secondary structures within regions of template DNA during the extension process (44). Such cosolvents may also act as destabilizing agents, reducing the melting temperature of GC-rich sequences, or as osmopro-

tectants, increasing the resistance of the polymerase to denaturation (44, 83).

Variations in Methodology To Improve Sensitivity and Specificity

A straightforward solution to difficulties encountered in the development of multiplex PCR has been the use of hot start PCR (21) and/or nested PCR (123). The former often eliminates nonspecific reactions (particularly production of primer dimers) caused by primer annealing at low temperature (4 to 25°C) before commencement of thermocycling (21). The procedure has recently been made more practicable through the use of a nonmechanical hot start methodology which involves the use of a form of *Taq* polymerase, for example, Amplitaq Gold (Roche Diagnostics), which is activated only if the reaction mixture is heated at approximately 94°C for 10 min (the first denaturation step) (8, 53). Nested PCR increases the sensitivity and specificity of the test through two independent rounds of amplification using two discrete primer sets. Although this adaptation is undoubtedly effective in most cases, it also considerably complicates the practical application of PCR. The second round of amplification delays results, increases the possibility of cross-contamination, and may complicate automation.

General Considerations for Multiplex PCR Development

Development of multiplex PCRs should follow a rational approach for the inclusion or exclusion of specific pathogens in the assay. These pathogens can be organ system specific or symptom specific with respect to the age of the patient and the epidemiological characteristics of these pathogens. PCR conditions, such as compatibility among the primers within the reaction mixture such that there is no interference, is of great technical importance. The primer pairs must be inclusive for as many strains of the target pathogen as possible, and depending on the amplicon detection method, their targets are easily resolvable. The latter may be achieved by using primer pairs that result in PCR products that can be separated and clearly visualized using gel electrophoresis or hybridization probes with maximum specificity. Prior to application in a clinical setting, multiplex PCRs must be evaluated for their sensitivity as compared with their corresponding uniplex PCRs using both serial dilutions of the target DNA and clinical specimens.

Wherever possible, multiplex PCRs should avoid the use of nested primers requiring a second round of amplification. The latter is a major contributor to false-positive results due to carryover contamination, although anticontamination protocols including PCR controls (reaction and specimens extraction controls) must be implemented in all PCR-based protocols (55). Likewise, precautions and methodologies to avoid false-negative results due to reaction failure have to be considered (104). Multiplex PCRs that amplify target sequences along with the presence of external or internal control target nucleic acids to indicate reaction failure have been developed (49, 62, 69).

APPLICATION OF MULTIPLEX PCR IN DIAGNOSTIC VIROLOGY

During the last decade, a number of studies have demonstrated the practicality of identifying viral pathogens in many clinical and epidemiological settings using multiplex PCR (Table 2). The technique has been used to screen for individual or symptom-associated viruses and examine associations of virus infection with disease. In addition, the technique has been shown to be a powerful and cost-effective tool for typing and subtyping virus strains in different epidemiological studies.

Neurotropic Viruses

PCR has proved to be a powerful tool for investigating meningitis and encephalitis caused by a variety of viruses. In neurological disease the requirement of rapid and reliable diagnosis to provide a rational basis for chemotherapy and limit unnecessary procedures and irrelevant therapy has driven development. The wide range of viruses associated with neurological disease includes herpes simplex virus (HSV); cytomegalovirus (CMV); varicella-zoster virus (VZV); Epstein-Barr virus (EBV); human herpes virus 6 (HHV-6); the enterovirus group, including echoviruses, polioviruses, and coxsackieviruses; adenoviruses; JC and BK viruses; arenaviruses; paramyxoviruses; rabies; and arboviruses. In view of the large number of potentially neuroinvasive viruses and because of the limited volume of the most useful diagnostic specimen—cerebrospinal fluid (CSF)—a number of multiplex PCRs have been developed (12–15, 82, 102).

The feasibility of simultaneous screening for viruses, bacteria, and parasites in CSF specimens from patients with aseptic meningitis or encephalitis has been described (82). This study by Read et al. (82) utilized three nested multiplex PCRs for detection of HSV and VZV; EBV and HHV-6; and members of the enterovirus group and echovirus type 22 and 23. In addition, two uniplex PCRs were used for detection of CMV and JC virus. In a total of 2,233 CSF specimens from 2,162 patients, the PCR was positive in 147 specimens from 143 patients (6.6% of all patients) including enteroviruses (77 patients), HSV-1 (20 patients), VZV (7 patients), HSV type 2 (HSV-2) (6 patients), CMV (3 patients), JC virus (2 patients), and HHV-6 (1 patient). All PCR assays remained negative with 28 control CSF specimens. The clinical sensitivity and specificity of this PCR were not determined because full clinical information was not available for all of the patients.

A nested multiplex PCR for detection and differentiation of HSV-1 and -2 on the basis of PCR product size has also been described (14). In a prospective analysis, a total of 417 CSF specimens obtained from 395 consecutive patients with clinical suspicion of HSV encephalitis, meningitis, or meningoencephalitis were tested by multiplex PCR. The test was positive for HSV-1 in 11 specimens (2.6%) from 10 patients and for HSV-2 in 4 specimens (1.0%) from 3 patients; no coinfection with both types was reported. The same multiplex PCR was used to test a total of 178 CSF samples obtained from 171 patients with clinical suspicion of herpes virus infection (15). The assay was positive for HSV-1 in three samples (1.7%) from two patients (1.2%) and for HSV-2 in one sample, and one patient tested positive in a nested uniplex CMV PCR. A similar procedure to detect the DNA of both viruses (HSV-1 and -2) was applied to CSF samples from 918 human immunodeficiency virus (HIV)-infected patients with neurological symptoms (22). In patients for whom a diagnosis was confirmed at autopsy, the test was positive for HSV-1 or -2 for 19 patients (2%), producing a sensitivity and specificity of 100 and 99.6%, respectively.

The first nested multiplex PCR for detection and typing of herpesviruses (HSV-1 and -2, VZV, CMV, HHV-6, and EBV) was applied to CSF from patients with meningitis, encephalitis, and other clinical syndromes (102). By utilizing equimolar concentrations of primers aligning the 3′ ends with one of two consensus regions within the herpesvirus DNA polymerase gene and the 5′ ends with the related or nonrelated sequences of each agent to be amplified, the first round of amplification yielded a 194-bp fragment indicating the presence of herpes-

TABLE 2. Application of multiplex PCR for diagnosis of viral infections

Clinical manifestation(s)	Specimen(s)	Viruses and/or other agent(s) targeted	Reference(s)
Meningitis, encephalitis, and/or meningo-encephalitis	CSF	HSV-1, HSV-2, and CMV	14
		HSV and VZV; EBV and HHV-6	81
		HSV-1, HSV-2, VZV, CMV, HHV-6, and EBV	102
		HSV-1 and HSV-2	15
		HSV-1, HSV-2, VZV, CMV, HHV-6, EBV, and EVs[a]	12, 13
		CMV, EBV, HHV-6, HHV-7, and HHV-8	77
		EBV and *T. gondii*	87
Upper and lower respiratory infections	Throat, nose, and nasopharyngeal swabs; nasopharyngeal and endotracheal aspirates; bronchoalveolar lavage	Influenza viruses A and B	30
		PIV types 1, 2, and 3	27
		Influenza virus and RSV	98
		RSVs A and B, influenza viruses A and B, PIV types 1, 2, and 3	33
		RSV, PIVs, adenovirus	74
		EV, influenza viruses A and B, RSV, PIV types 1 and 3, adenovirus, *M. pneumoniae*, *C. pneumoniae*	36
Conjunctivitis, keratitis, keratoconjunctivitis	Conjunctival and corneal swabs	HSV-1 and HSV-2	15
		Adenovirus and HSV	49
		Adenovirus, HSV, and *C. trachomatis*	Yeo et al.[6]
Genital ulcer disease	Genital ulcer swabs	HSV, *H. ducreyi*, and *T. pallidum*	7, 66, 73
Genital lesions	Lesion and endocervical swabs	HPVs, HSV, and *C. trachomatis*	64
HPV-associated genital disease	Cervical scrapings, smears, and biopsies; vaginal and vulval swabs	HPVs	23, 35, 56, 75, 97, 123
Vesicular rashes	Vesicle fluids	HSV and VZV	3
Hepatitis	Serum and plasma samples	HBV genotypes	84
		HCV, HGV, and GB viruses	16
Immunocompromised status	Plasma	HHV-6 and HHV-7	67
	Blood	HIV-1, HIV-2, HTLV-1, HTLV-2	45

[a] EVs, enteroviruses.
[b] Abstr. 97th Gen. Meet. Am. Soc. Microbiol., 1997.

virus. The second round of amplification utilizing primer mixtures contained nonhomologous and type-specific primers selected from different regions of the aligned DNA polymerase genes of human herpesviruses produce a product with a different size for each related virus. The method amplified the corresponding virus in infected cells and in five clinical samples (HSV-1 PCR-positive CSF from a patient with encephalitis, HSV-2 PCR-positive CSF from a patient with meningitis, VZV culture-positive vesicular fluid from a patient with shingles, CMV culture-positive urine from a congenitally infected patient, and EBV PCR-positive peripheral blood from a patient with a lymphoproliferative syndrome). In addition, the use of primers targeting consensus regions may allow recognition of new, undescribed human herpesviruses. The detection of a 194-bp fragment after the first reaction with no positive signal in the second round of amplification could reflect the detection of a new human herpesvirus. The test was further modified to include a reverse transcription step and primer pairs to detect enterovirus cDNA (12). This PCR was then evaluated in 21 patients with etiologically well-characterized aseptic meningitis and encephalitis. HSV DNA was detected in nine patients, VZV DNA was detected in 6 patients, and enterovirus RNA was detected in 6 patients. The test was further evaluated for detection of these same viruses in CSF samples by a prospective study of 200 neurological-disease patients suspected to have viral infections. Enterovirus was detected in 49 patients, HSV was detected in 3 patients, VZV was detected in 6 patients, CMV was detected in 12 patients, EBV was detected in 2 patients, CMV and HSV were detected in one AIDS patient with encephalitis, and CMV and EBV were detected in another AIDS patient with polyradiculomyelitis. For detection of echovirus 30 in 50 patients with aseptic meningitis, the multiplex reverse transcription (RT)-PCR was more sensitive (90% sensitivity) than cell culture (26% sensitivity) and the Amplicor EV test (86% sensitivity). These studies demonstrate the utility of this multiplex RT-PCR for detection of enteroviruses and herpesviruses in CSF samples from patients with various neurological manifestations and the usefulness of the technique in patient management and design of antiviral therapy.

In the United Kingdom, a nested multiplex PCR for the detection of HSV-1 and -2, VZV, and enteroviruses, the four most common causes of viral meningitis and encephalitis, was developed and evaluated using a total of 1,683 consecutive CSF samples (81). The test was positive in 138 (8.2%) of the specimens (enteroviruses in 51 samples, HSV-2 in 33 samples, VZV in 28 samples, and HSV-1 in 25 samples). Of the 51 patients positive for enterovirus RNA, 17 were babies less than 6 months old in whom the CNS infection was detected as part

of a general infection screen and 34 patients were older children and adults who had encephalitis and meningitis. In the group positive for VZV (28 patients), 16 patients had meningitis and 10 had encephalitis but clinical details were not available for 2 patients. HSV-1 was detected in two babies less than 6 months old and in 23 adults (22 had encephalitis and 1 had a benign lymphocytic meningitis). The HSV-2-positive patients (33 patients) included five babies less than 6 months old, two adults with meningoencephalitis, and 26 patients more than 6 months old with benign lymphocytic meningitis. These tests proved suitable for routine use in a diagnostic laboratory and highlighted the importance of screening for more than one virus in patients with meningitis and encephalitis.

Although the studies described above (13–15, 81, 82, 102) produced satisfactory results in terms of simultaneous screening for neurological manifestation-associated viruses (for example herpesviruses), the multiplex PCRs developed utilized a nested strategy. The latter as described earlier may increase the chance of false-positive results due to contamination and may also complicate automation. A recent study (63) utilized a PCR assay which precludes the use of nested primers for simultaneous amplification of herpesviruses DNAs. This assay, termed consensus PCR, uses a pair of "stair" primers, which are based on consensus sequences selected from within the DNA polymerase gene and were 76 to 86% identical to the genomic sequences of the six herpesviruses (HSV-1, HSV-2, CMV, EBV, VZV, and HHV-6) that may infect the CNS. Each stair primer used comprised an equimolar mixture of 11 oligonucleotides corresponding to a consensus sequence: all primers had the same 5′ end but extended for 20 to 30 nucleotides in the 3′ direction. The PCR products were analyzed by hybridization in microtiter plates using virus-specific, biotinylated oligonucleotide probes. The consensus PCR was evaluated using 142 CSF samples previously tested by standard uniplex PCRs. Eighteen samples (12.7%) tested positive by the uniplex PCRs, and 37 (26%) tested positive by the consensus PCR, including 3 samples that had coinfections (CMV, VZV, and HSV-2; VZV and HSV-2; and CMV and HHV-6). Of the 142 CSF samples, 103 were classified as negative by both the uniplex PCRs and the consensus PCR. In addition, the test showed high diagnostic utility in that several cases were found to be positive for viruses for which tests were not requested by the clinician.

The problem in evaluating all of the aforementioned multiplex PCR studies is that most have not included complete patient detail. Thus, while many positive results have been related to compatible clinical illness, positive results are not reported in all patients with similar conditions. Nevertheless, the multiplex PCR detects more positive specimens and is more rapid than conventional techniques such as culture or serology. However, the latter procedures are either insensitive or slow and make an unsatisfactory yardstick ("gold standard") against which to measure the accuracy of multiplex PCR. Because uniplex PCR has more data available and thus is better substantiated as to its clinical value, results of multiplex PCR should be compared with those of uniplex PCR to ensure that multiplex PCR has equivalent sensitivity, specificity, and clinical relevance.

Respiratory Viruses

Viruses that commonly cause respiratory infection include respiratory syncytial virus (RSV), influenza viruses and parainfluenza viruses (PIV), and adenovirus, especially in infants and young children. Infection with these viruses may result in severe lower or upper respiratory tract disease requiring hospitalization. Thus, sensitive and rapid testing for these viruses is crucial to reduce the potential of nosocomial transmission to high-risk patients, limit unnecessary antibiotic use, and direct appropriate therapy following a specific diagnosis (119). For this reason, a number of studies have aimed to develop and evaluate multiplex PCR for detection of these viruses and provided substantial evidence of the utility of this technique as an important tool for management of patients presenting with respiratory infections. A number of studies have utilized multiplex PCR to both detect and type or subtype influenza viruses, PIVs, and RSV in clinical specimens and are summarized below.

A nested multiplex RT-PCR which included three primer pairs in each round of amplification was utilized for the simultaneous detection, typing, and subtyping of influenza type A (H3N2 and H1N1) and type B viruses in a prospective surveillance of influenza in England in the 1995–1996 winter season (30). A total of 619 combined nose and throat swabs from patients with an influenza-like illness were analyzed by culture and multiplex PCR. The multiplex RT-PCR detected influenza viruses in 246 (39.7%) samples compared to the 200 (32.3%) which yielded influenza viruses in culture. In addition, there was excellent correlation between the multiplex RT-PCR and culture for typing and subtyping of influenza viruses (100%) and for temporal detection of influenza A H3N2 and H1N1 viruses. It was concluded that whereas the multiplex RT-PCR demonstrated its utility in detection of influenza viruses in patients with influenza-like illness, patients with influenza-like illness who are negative for influenza viruses may harbor a pathogen(s) producing a syndrome difficult to distinguish clinically from true influenza (for example, RSV). Indeed, when this multiplex RT-PCR was modified so that it was capable of detecting and subtyping influenza A (H1N1 and H3N2) and B viruses as well as RSV subtypes A and B in respiratory clinical samples (98), the assay again demonstrated excellent (100%) correlation with the results of culture and serology. The ability of the test to detect viral coinfection in both simulated specimens and clinical samples was also demonstrated.

A nested multiplex RT-PCR using three primer pairs was developed to detect PIV types 1, 2, and 3 in throat and nasopharyngeal swabs (27). In the first round of amplification, similar-size fragments are produced. In the second round of amplification a series of three internal primer pairs are introduced, producing type-specific amplicons that were easily differentiated based on size upon gel electrophoresis. The test detected and correctly typed PIV in 15 isolates and 26 of 30 (87%) previously positive nasopharyngeal specimens but remained negative in naso- or oropharyngeal specimens and/or culture isolates of 33 unrelated respiratory tract pathogens. In a modified version, the test was also used to detect RSV and adenovirus utilizing five primer sets to amplify cDNA of RSV subtypes A and B; PIV types 1, 2, and 3; and DNA of adenovirus types 1 to 7 (74). The test was sensitive and specific for all 12 tissue culture-grown prototype viruses and when applied to respiratory specimens was more sensitive (41 of 112) than direct immunofluorescence or antigen detection following culture (34 of 112). Among positive samples, multiple respiratory viruses were found in four specimens, further illustrating the potential utility of this multiplex PCR assay.

A multiplex quantitative RT-PCR enzyme hybridization assay (Hexaplex; Prodesse, Inc., Milwaukee, Wis.) which combines primers originating from highly conserved regions of 7 respiratory viruses (RSV subtypes A and B; PIV types 1, 2, and 3; and influenza viruses A and B) with probes for the detection of PCR products using enzyme hybridization assay has also been described (33). The assay provides rapid simultaneous

detection, identification, and quantitation of these viruses in nasal wash specimens in a single test. The primer and the probes utilized were evaluated using multiple virus isolates from each group and resulted in specific PCR products from all tissue culture-positive specimens without cross-reactivity among these seven viruses or with other common human respiratory viruses. The Hexaplex assay was applied on nasal wash specimens from 69 children with signs of lower respiratory tract infection and 40 specimens from asymptomatic children. Of the 69 specimens from symptomatic children, 37 were positive by the Hexaplex assay but only 29 of these were culture positive. Both the Hexaplex assay and virus culture were negative in the 40 nasal washes from the asymptomatic children. The Hexaplex assay was 100% sensitive and 98% specific in comparison with virus culture.

Multiplex PCR in combination with a heteroduplex mobility shift assay has proved to be a valuable and cost-effective tool for monitoring the emergence of new variants or new subtypes of influenza viruses arising through the phenomena of antigenic drift and antigenic shift (125, 126). On the basis of amplicon size, the amplification assay differentiates the variable region of the hemagglutinin genes of the H1 and H3 subtypes of influenza viruses A and B and the counterpart (hemagglutinin, esterase, and fusion gene) of influenza virus C. Variants within the same type or subtype are then identified by heteroduplex mobility shift assay of the amplicons. This approach proved to be a rapid, sensitive, and reliable method for the detection and typing of influenza virus and for screening for influenza virus variants, proving capable of identifying new influenza B virus variants (126).

More recently, it has been demonstrated that multiplex PCR is a useful and rapid diagnostic tool for the management of children with acute respiratory infections (36). This simplified hot start multiplex PCR allows simultaneous screening for nine different infectious agents (enterovirus, influenza viruses A and B, RSV, PIV types 1 and 3, adenovirus, *Mycoplasma pneumoniae*, and *Chlamydia pneumoniae*). The test was evaluated using clinical samples from 1,118 children with acute respiratory infections and was positive in 395 (35%) samples. Of these, 37.5% were positive for RSV, 20% were positive for influenza A virus, 12.9% were positive for adenovirus, 10.6% were positive for enterovirus, 8.1% were positive for *M. pneumoniae*, 4.3% were positive for PIV type 3, 3.5% were positive for PIV type 1, 2.8% were positive for influenza B virus, and 0.2% were positive for *Chlamydia trachomatis*. Seasonal variations in the rates of detection of the different organisms were noted. The test demonstrated levels of concordance of 95% for RSV and 98% for influenza A virus with the data obtained by commercially available enzyme immunoassay.

Genito-Urinary Infections

The utility of multiplex PCR in diagnosis of viruses associated with genital tract infection is reflected by the numerous reports detecting and typing human papilloma viruses (HPVs). These multiplex PCRs have a variety of formats but with a common aim of detecting and typing HPVs. These include PCRs which combined primers to produce a type-specific product size (97, 103) or those that utilized degenerate primers for HPV screening and strain-specific antisense primers for simple typing (56). In an alternative protocol, three consensus primer pairs simultaneously detected high-risk (for cervical carcinoma) HPV type 16 (HPV-16) and HPV-18 and low-risk HPV-6 and HPV-11, and primer pairs for more than 40 other HPV types were developed (consensus multiplex PCR) (124). This approach was considered to be a consensus multiplex PCR assay because all the primers are consensus and are multiplexed in the same PCR mixture for simultaneous amplification. One primer pair amplifies general HPV DNA from more than 40 types, including HPV-6, -11, -16, and -18 (450-bp PCR product), indicating HPV infection. Another primer pair generates a PCR product of 307 bp if HPV-16 and -18 DNA are present in the sample (high-risk HPV infection). The third consensus primer pair results in a PCR product of 550 bp with low-risk HPV infection (HPV-6 and -11). The assay was evaluated on exfoliated cell specimens obtained from 148 healthy women, biopsied specimens from 32 patients with condyloma acuminata, and biopsied specimens from 76 patients with invasive carcinoma of the uterine cervix. The multiplex PCR was positive in 47 (31.8%) of the samples from the 148 healthy women, but most of the positive samples were in samples from women infected with HPV types other than HPV-6 and -11 or HPV-16 and -18. All samples from patients with condyloma acuminata contained HPV DNA but mainly contained HPV-6/11 (87.5%), while high-risk HPV had a low prevalence rate (6.5%). HPVs were detected in samples from 69 (90.8%) of the 76 patients with cervical carcinoma, of which high-risk HPVs accounted for 82.9%. The method proved to be a simple, economical, and reliable tool for detection of HPV infection. The simultaneous amplification of the three HPV targets should allow rapid approach for distinguishing disease-related HPV types: low-risk HPVs (HPV-6 and HPV-11) involved in genital, benign lesions, such as warts or condylomas, and high-risk HPVs (HPV-16 and HPV-18), which are frequently found in malignant lesions of the lower genital tract. This should provide valuable information for monitoring and treating patients with HPV-related lesions, although the method lacks the capability to define individual HPV types, which limits its usefulness in epidemiological investigations.

Three consensus primers (two sense primers and 1 antisense primer labeled with dinitrophenyl) were used in a multiplex PCR assay for detection and typing of oncogenic and nononcogenic HPV types (23). The amplification products were hybridized with specific labeled oligoprobes mixed in two cocktails (oncogenic and nononcogenic biotinylated HPV oligoprobes) which could then be deposited in one well of streptavidin-coated microplates. PCR products were detected with anti-dinitrophenyl monoclonal antibody and horseradish peroxidase (PCR-enzyme immunoassay). The test was evaluated in cervical scrapings from 181 patients at high risk for cervical cancer selected because of their histories of cytological and/or histological cervical and vaginal abnormalities. These patients were classified with regard to the presence of lesions in cervical scrapings as none (i.e., no lesions; $n = 137$), low grade (i.e., few lesions $n = 20$), and high grade (i.e., many lesions $n = 24$). In patients without lesions, the multiplex PCR detected nononcogenic HPV in 29 patients (18.3%) and oncogenic HPV in 44 patients (32.1%), including 21 (15.3%) patients presenting with coinfection. In the low-grade group, oncogenic HPV was detected in 12 patients (60%) and nononcogenic HPV was detected in 5 patients (25%), including 4 (20%) cases of coinfection. In the high-grade patients, oncogenic HPVs were detected in 95.8% (23 patients) with coinfection with nononcogenic HPV in 4 of these patients (16.7%). The multiplex PCR was negative in scrapings from the remaining 93 patients (85 patients without lesions, 7 patients in the low-grade group, and 1 patient in the high-grade group). The authors concluded that the test is simple and reproducible and can be automated.

Another variation (75) combined a nested multiplex PCR, utilizing primers specific for low- or high-risk HPV types with restriction endonuclease analysis. The accuracy of this ap-

proach was confirmed by examining cervical scrapings from 44 patients. HPVs were detected and typed in scrapings from 7 patients with koilocytosis, 8 patients with dysplasia or metaplasia, 3 patients with condyloma acuminata, and 18 patients with cervical invasive neoplasia but in no samples from the seven healthy controls. More recently, a simple multiplex PCR utilizing three primer pairs for amplification of HPV-16, -18, and -33 in combination with a colorimetric microplate hybridization as a post-PCR detection system has been developed (35). The system uses three type-specific capture oligonucleotides linked covalently to a single microplate well and three type-specific multibiotinylated probes for detection. The methodology was evaluated using a total of 55 cervical smears and biopsy samples from 55 women with cervical lesions, resulting in 100% correlation with the results of other PCRs using consensus primers.

A multiplex PCR that combined the detection of *C. trachomatis* and two viruses (HPV and HSV) was developed, optimized, and evaluated using cervical and endocervical specimens from patients suspected to be infected with one or more of these agents (64). The test produced 100% correlation with the results of the uniplex PCRs in 92 genital swabs (29 were positive for HSV, 16 were positive for HPVs, and one was positive for *C. trachomatis*). In addition, a coinfection with HPV and HSV was detected by the multiplex PCR. In other studies (7, 66, 73), primer pairs for HSV were combined with those for *Haemophilus ducreyi* and *Treponema pallidum* to construct a multiplex PCR for diagnosis of genital ulceration. The PCR products were detected utilizing a colorimetric detection system in which three separate microwells containing immobilized oligonucleotide capture probes were used. In one of these studies (73), the sensitivity of this multiplex PCR in 298 genital ulcer swab specimens to detect HSV, *H. ducreyi*, and *T. pallidum* were 100, 98.4, and 91%, respectively, compared to 71.8, 74.2, and 81% by HSV culture, *H. ducreyi* culture, and darkfield microscopy for *T. pallidum*, respectively. The same multiplex PCR was used to evaluate swab specimens from 38 sequential patients with genital ulcer disease who received clinical diagnoses and syndromic treatment (7). These specimens were also tested for *H. ducreyi* by culture which was reported to be negative for all specimens tested. Of the 38 specimens, the multiplex PCR detected HSV in 31 specimens (81.6%) and HSV and *T. pallidum* in 1 specimen (2.3%) (coinfection). The test was negative in the remaining six specimens (15.8%). The clinical diagnoses corresponded poorly to the results of the multiplex PCR for chancroid and syphilis; none of the samples from the six suspected cases of chancroid and the one case of syphilis were positive for *H. ducreyi* and *T. pallidum*, respectively, by the multiplex PCR. Of the six clinically suspected cases of chancroid, the multiplex PCR was negative for all pathogens in one case and positive for HSV in the remaining five cases. Of 24 clinically suspected cases of genital herpes, 21 cases (87.5%) were confirmed by multiplex PCR.

Although clinical findings correlated poorly with multiplex PCR results, it is known that the clinical manifestations of all three infections can vary significantly (25). A few specimens produced negative results for which there may be several possible explanations, including ulcers due to trauma, low organism numbers, inadequate sampling techniques, and lesions due to other etiologic agents. These studies highlight the advantages of multiplex PCR over standard laboratory techniques and allow the detection of coinfection.

Ocular Infections

The benefits of PCR diagnostics over conventional techniques in the diagnosis of ocular infection are well documented for both the anterior and posterior segment of the eye (1, 24, 31, 80). The development and evaluation of multiplex PCR for detection of adenovirus, HSV, and *C. trachomatis* in cases of keratoconjunctivitis demonstrated the feasibility of simultaneous screening for these agents (49; A. C. Yeo, R. J. Cooper, D. J. Morris, and C. C. Storey, Abstr. 97th Gen. Meet. Am. Soc. Microbiol., abstr. C-416, 1997). These studies further highlight the difficulties of multiplex PCR and also provide substantial evidence for the importance of careful selection of oligonucleotide primers. In the study by Jackson et al. (49), a multiplex PCR was designed to detect adenovirus and HSV in eye swabs. The test produced results identical to those of virus isolation for 18 of 20 eye swabs (positive for adenovirus in five swabs, positive for HSV for five swabs and negative for adenovirus and HSV in eight swabs) but the remaining two specimens positive for adenovirus and HSV by virus isolation were negative by the multiplex PCR. However, the multiplex PCR proved superior to culture for the rapid diagnosis of viral keratoconjunctivitis. Replacement of the adenovirus primer pair to allow broader reactivity with adenovirus serotypes and inclusion of a primer pair targeting the cryptic plasmid of *C. trachomatis* (A. C. Yeo, R. J. Cooper, D. J. Morris, and C. C. Storey, Abstr. 97th Gen. Meet. Am. Soc. Microbiol., abstr. C-146, 1997) yielded a triplex multiplex PCR. The sensitivity of this adenovirus-HSV-*C. trachomatis* multiplex PCR in comparison to a uniplex PCR for the detection of adenovirus was 100%. However, the performance of the test to detect either HSV or *C. trachomatis* was relatively poor (69% compared to cell culture or 72% compared to an antigen detection technique). Selection of alternate HSV and *C. trachomatis* primer pairs allowed development of a multiplex PCR with identical sensitivity to that of uniplex PCRs for detection of each of the three targets.

Immunocompromised Patients

Due to the importance of HHV infections in immunocompromised patients, a number of authors have developed methods for the simultaneous detection of these viruses in various clinical specimens. Two different genes of CMV were detected with a single-step multiplex PCR in clinical specimens from renal transplant recipients and other CMV-seropositive patients (11). The test was sensitive and allowed monitoring of CMV infection by quantitation of CMV DNA. The latter was based on the assumption that samples with small amounts of viral DNA are more likely to lead to the amplification of only one of the two targets. Samples from which a single target is amplified contain on average sevenfold fewer viral genomes per 10^6 leukocytes than those from which both targets are amplified. This approach was evaluated on serial leukocyte DNA samples taken from 34 patients during the first 12 weeks post-renal transplantation, and it was concluded that findings of three consecutive tests in which both CMV targets were amplified were highly indicative of patients who had developed a very high load of CMV with a sensitivity of 100% and specificity of 88%. Thus, the protocol can be used for efficient identification of transplant recipients at risk of clinically significant infection. A similar PCR format was used to develop a multiplex PCR for amplification of four different regions of the CMV genome (61). The test sensitivity for detection of genomic cell DNA infected with CMV was similar to that obtained by each pair of the primers (uniplex PCRs). The test

proved to have a high diagnostic utility for detection of CMV variants with maximal sensitivity and specificity.

In heart transplant recipients a hot start nested multiplex PCR was used to evaluate the possible reactivation of HHV-6 and HHV-7 (67). The test was also coupled with CMV antigenemia assay and HSV isolation. The multiplex PCR was performed on buffy coat and plasma samples from the 21 recipients and on buffy coat samples from healthy blood donors. Whereas 12 of 21 (57.1%) heart transplant recipients showed CMV and/or HSV reactivation, HHV-6 was detected in 2 of 21 (9.5%) of the recipients and in 7 of 56 (12.5%) blood donors. HHV-7 DNA was detected in 13 of the 21 recipients (61.9%) and in 30 of 56 (56.6%) blood donors. One of the patients positive for HHV-6 and 10 of those positive for HHV-7 were positive for CMV. The clinical significance of these concurrent infections remains to be determined, but their detection further highlights the utility of multiplex PCR in investigation of transplant patients.

Detection and typing of all human lymphotropic herpesviruses (EBV, CMV, HHV-6 variants A and B, HHV-7, and HHV-8) utilizing primer pairs designed to amplify a highly conserved region within the DNA polymerase gene have also been described (77). The test also included an internal control (100 molecules of a cloned fragment of the porcine pseudorabies herpesvirus genome) for detection of false-negative results. The test revealed a sensitivity of 10 to 100 molecules of each virus DNA and produced a 100% correlation in a total of 35 well-characterized specimens in which one of these viruses was known to be present, including Kaposi's sarcoma skin lesions and serum, CSF, saliva, and urine samples.

Simultaneous detection of EBV and the protozoa *Toxoplasma gondii* in CSFs of AIDS patients with EBV-associated primary nervous system lymphoma and toxoplasma encephalitis has been described (87). This multiplex PCR detected EBV DNA in 9 of 14 patients with CNS lymphoma and in 2 of 38 patients without disease and *T. gondii* DNA in 8 of 8 patients with toxoplasma encephalitis but in none without toxoplasmosis.

The major problem with the use of PCR in specimens from immunocompromised patients is the relationship of positive results to clinical disease. This is particularly difficult with herpesviruses, because positive PCR results may not always be accompanied by signs or symptoms in the patient. This can leave the clinician in a dilemma of whether or not to treat. Two approaches have been used to address this problem. Quantitative PCR can be used to measure viral load with the expectation that a high viral load is likely to herald clinical disease. The other approach is to test sequential specimens and only recommend treatment if positive results persist rather than responding to what may be a transient reactivation of a herpesvirus. Multiplex PCR may be readily applied to the latter situation, but multiplex quantitative PCR remains a formidable technological challenge.

Other Applications

A number of studies have utilized multiplex PCRs for detection and differentiation of human retroviruses (45, 46, 99, 111). Four primer pairs were combined to detect the *gag* region of HIV type 1 (HIV-1), the *env* region of HIV-2, the *pol* region of human T-cell leukemia virus type 1 (HTLV-1), and the *tax* region of HTLV-2 (45). Amplicons were detected by liquid hybridization using ^{32}P-end-labeled oligonucleotides. In the evaluation of a serologically well-established panel of singly and dually infected individuals, the assay detected 21 of 22 HIV-1, 8 of 10 HIV-2, 8 of 8 HTLV-1, and 8 of 8 HTLV-2 infections. The test was as sensitive as uniplex PCRs and allowed the detection of coinfection. Sunzeri et al. (99) developed a multiplex PCR utilizing primer pairs targeting a portion of the *gag* region of HIV-1, the *pol* gene of HTLV-1 and -2, and a region of the HLA-DQ-α locus as an internal control. Products were analyzed by automated capillary DNA chromatography (products can also be separated and visualized using gel electrophoresis and ethidium bromide staining). The test detected as few as 1 to 10 infected cells (2 to 20 target sequences) and was as sensitive as uniplex PCRs.

Several studies have highlighted the potential use of PCR in screening donated blood for transfusion-transmitted viruses (48), but the need for multiple, discrete PCR assays for achieving this purpose has restricted such an application. Uniplex PCR would be an impractical approach for rapid screening of hundreds of specimens per day for a range of transfusion-transmitted viruses. Multiplex PCR presents a more practicable solution to the problem, and the methodology is sensitive enough to diagnose silent (serologically negative) carriers of viruses such as HIV, HTLV, and other nonretroviral transfusion-transmitted viruses, including hepatitis B virus (HBV), HCV, and CMV.

Multiplex PCR offers a cost-effective solution which, with refinement and full automation would allow screening of the donated blood supply. Indeed, a recent study (106) demonstrated the promise of multiplex PCR automation for both rapid and reliable screening of transfusion-transmitted viruses in donated blood and transplantable tissues. A hot start multiplex PCR was developed to identify and determine the abundance of HIV-1, HIV-2, and HTLV-1 and -2. Viral DNA sequences were amplified in a single reaction, and the resulting amplicons were detected in real time by the hybridization of four differently colored, amplicon-specific detector probes called molecular beacons present within the same reaction tube. The color of the fluorescence produced during the amplification process identified the retrovirus present in the sample, and correlation of the thermal cycles required with the intensity of each fluorescent signal developed provides an accurate measure of the number of virus sequences present in the original sample. The test had a sensitivity of 10 retroviral genomes in the presence of 100,000 copies of another retrovirus, and up to 96 samples can be analyzed in 3 h on a single plate. The test was also evaluated using 43 human blood samples known to contain human retroviruses and produced 100% agreement in 11 samples positive for HIV-1, 4 samples positive for HIV-2, 15 samples positive for HTLV-1, and 17 samples positive for HTLV-2, and the 10 control samples remained negative for all targets.

Multiplex PCRs for other transfusion-transmitted viruses have also appeared in the literature. A multiplex PCR which detects both HBV and HBC genomic sequences in serum samples has been developed (71). The test is carried out in two stages. HCV RNA is first reverse transcribed into cDNA, and both HCV cDNA and HBV DNA are then coamplified using primers that target conserved sequences from both viruses. The test was applied to sera from nine donors, of which seven were positive for HBsAg, anti-HBc, and anti-HCV; one was reactive for both anti-HCV and anti-HBc; and one was reactive for both HBsAg and anti-HBc. The multiplex PCR produced results confirming the presence of both HBV- and HCV-specific genomic sequences in eight of eight sera reactive for the serological markers of both viruses and also in a serum that was reactive for HBV markers only. The method was modified to include primers that target the HCV 5′ untranslated region and HBV pre-S and S region in a one-step multiplex PCR method. This modification allowed a simple simultaneous am-

plification of both viruses with 100% concordance with its respective uniplex PCRs (47).

HCV and GB virus type C (GBV-C)/HGV genomes in plasma samples from transfused subjects have also been amplified by a multiplex PCR (16). The test was evaluated retrospectively in 50 plasma samples in comparison with the results of serology. Of the 50 samples, 40 were positive for anti-HCV and 10 samples remained antibody negative. The multiplex PCR and the corresponding uniplex PCR produced identical results, being positive for HCV RNA in 32 samples of the 40 anti-HCV positive samples. GBV-C RNA was detected in 5 of the 32 HCV-positive samples (coinfection) and in 2 of 10 samples that were anti-HCV negative. More widespread application of multiplex PCR aimed at detecting transfusion-transmitted viruses and/or other pathogens will demand thorough optimization and full automation of procedures to avoid the production of both false-positive and false-negative results. The demands of transfusion medicine of high-speed, high-throughput screening will place great technical demands upon these procedures.

Multiplex PCR methodology has also proven to be a valuable tool for differentiation, subgrouping, subtyping, and genotyping of viruses (10, 29, 78, 84, 121). Differentiation of polioviruses from nonpoliovirus enteroviruses in both clinical (stool) and environmental (sewage) specimens has been feasible using RT-multiplex PCR (29). Stool or sewage specimens are first inoculated onto cell cultures in tubes, and after overnight incubation the cultures are subjected to RT-multiplex PCR. A primer pair detecting all enteroviruses in the presence of another two primer pairs specific for the polioviruses was tested. The enterovirus-specific primer pair generated a product size of 300 bp, and the poliovirus-specific primer pairs generated three different PCR products of 200, 600, and 1,000 bp, assuring easy identification on agarose gels. The result is interpreted as "nonpolio enterovirus" if only the enterovirus-specific PCR product (300 bp) is observed; as "poliovirus" if both the enterovirus-specific product (300 bp) and at least one of the polio-specific products are observed (200, 600, and 1,000 bp); or as "no enterovirus" if the multiplex PCR remains negative for all products. A total of 36 poliovirus strains produced the expected results by the multiplex PCR, consistent with polioviruses, and only one isolate (coxsackievirus A21) of 45 nonpoliovirus strains demonstrated a band with a poliovirus-specific primer pair. The protocol revealed a sensitivity of 3 PFU, could be interpreted within 24 h, and was highly insensitive to substances in the sample (stool and sewage) which inhibit cell culture isolation.

A multiplex RT-PCR that simultaneously identifies Sabin poliovirus types 1, 2, and 3 vaccine strains has been described (10). This hot start-based multiplex PCR was a modification of a procedure previously described (121). The assay utilized three serotype-specific primer sets that map to the region of the poliovirus genome encoding the amino terminus of the VP1 capsid protein, a region known for its heterogeneity among the three Sabin poliovirus serotypes. In a total of 195 stool samples collected from 26 vaccinees following administration of the first dose of the trivalent oral vaccine, the multiplex PCR was more sensitive than culture for the detection of poliovirus types 1, 2, and 3. The percentages of specimens positive by the multiplex PCR for serotypes 1, 2, and 3 were 67.2, 82.6, and 53.8%, respectively, compared to 55.4, 64.1, and 27.7% by virus isolation. The duration of recovery of positive samples by PCR varied according to serotype: 4 to 8 weeks for type 2 and 1 to 8 weeks for types 1 and 3, although poliovirus type 3 shedding ceased in approximately 70% of vaccinees within a week after immunization. This modified multiplex PCR allows direct characterization of the virus in stool specimens without cell culture—a process which may, through the selection of genetic variants, not accurately represent the virus population in the original specimens.

Human adenoviruses in clinical samples were detected with six primer pairs specific for all adenovirus subgenera (A to F) (78). Each primer pair consists of a primer derived from the subgenus-specific sequences and a primer that targets a conserved hexon region, obviating the need for restriction endonuclease analysis. The six subgenus-specific amplicons were distinguishable by agarose gel electrophoresis as products of 299, 465, 269, 331, 399, and 586 bp representing, respectively, those of adenovirus subgroup A to F. The test revealed a detection limit of a single copy of adenovirus DNA. The primer pairs produced 100% specificity when evaluated on 23 adenovirus prototypes, representing all six subgenera; on 9 intermediate strains from subgenera B and D; and on 16 subgenus C genome types. In clinical specimens, the test was positive for adenovirus in 26 of 65 stool specimens (4 samples belonging to subgroup A, 2 samples belonging to subgroup B, 6 samples belonging to subgroup C, 13 samples belonging to subgroup F, and 1 sample showing coinfection with subgroup C and F), in 13 of 23 eye specimens (1 sample belonging to subgroup B, 10 samples belonging to subgroup D, and 2 samples belonging to subgroup E), and 2 of 12 throat specimens (belonging to adenovirus subgroup B). Of significance, the test has clinical value as can be highlighted by its discrimination of adenovirus subgroup D, which causes the severe and highly contagious epidemic keratoconjunctivitis, from subgroup B and E adenoviruses, which may cause relatively mild ocular infections. The test could also facilitate the primary classification of unknown virus isolates.

Multiplex PCR has also proved to be a valuable tool for genotyping HBV strains (84). The first round of amplification utilizes a primer pair to amplify the entire pre-S region of the virus genome. Within the pre-S region nucleotide exchanges are observed that are partly correlated to the serological surface antigen subtypes. In the second round of amplification, five additional subtype-specific primers and two universal non-group-specific primers are added to generate two to four DNA fragments of defined sizes indicative for the subtype. The method proved to be a useful epidemiological tool for studying HBV transmission and may be adapted to genomes of other infectious agents demonstrating a suitable degree of sequence variability.

Other applications of multiplex PCR include maximizing the inclusivity of PCR-based assays to detect viral strains (11, 51, 61, 93), exploring genetic reassortment among viruses (90), studying association of virus with disease (105), studying virus pathogenesis (6), exploring mechanisms of virus evasion and interference with the host's immune response (41), and facilitating the detection and characterization of viruses and other pathogens retrospectively in diverse archival specimens of limited volumes (2). In addition, the methodology has proved to be a powerful tool for characterization of nonhuman viruses (50, 70, 85, 104, 114, 116).

CONCLUSION AND PERSPECTIVES

Optimization of multiplex PCRs can prove difficult. A stepwise matrix-style approach may be followed; i.e., a number of optimal primer pairs are combined and the combination giving the best result is then chosen to be optimized or evaluated in a multiplex PCR format. Alterations of other PCR components over those usually described for most uniplex PCRs have rarely improved the efficiency of the test. Recent developments

in PCR technology, however, may facilitate the development of multiplex PCRs. The most appropriate of these seems to be the use of the nonmechanical hot start PCR (8, 53).

Thorough evaluation and validation of new multiplex PCR procedures is essential. The sensitivity and specificity must be thoroughly evaluated using standardized, purified nucleic acids. Where available, full use should be made of external quality control materials, and both external and internal quality controls must be rigorously applied. These must include the provision of both negative control specimens and, for each nucleic acid target, a positive control designed to ensure early signalling of any reduction in test sensitivity from assay to assay. As the number of microbial agents detectable by PCR increases, it will become highly desirable for practical purposes to achieve simultaneous detection of multiple agents that cause similar or identical clinical syndromes and/or share similar epidemiological features. In addition, attention should be given to primer pairs detecting multiple strains or types to ensure the identification of as many strains of the target species as possible. Where possible, robust (i.e., reliable in routine diagnostic settings) multiplex PCRs that do not use nested primers are preferable to avoid contamination (122), to rationalize use in routine settings, and to facilitate automation (110).

Commercial development of PCR has facilitated the widespread introduction of this procedure and improved both the reliability and ease of use of the technology. Commercially available applications of multiplex PCR are as yet in their infancy. While many commercially available PCRs include either internal control molecules or reporter molecules as internal standards within the test, the primer binding targets of both internal control and target nucleic acids are the same. Thus, although two molecules are amplified in the PCR, this is not true multiplexing. To date a multiplex PCR for *Neisseria gonorrhoea* and *C. trachomatis* represents the sole commercial multiplex PCR from Roche Diagnostics, and Argene Biosoft (Ariége, France) produces a multiplex PCR for detection of HHVs. Undoubtedly, many more commercial applications of multiplex PCR may be anticipated, and when coupled with developments in microelectronic detection devices (58) the prospect of extralaboratory "at-the-bedside" multiplex PCR testing may be envisaged. Given the advantages already demonstrated by the use of multiplex PCR along with the recent developments in this technology, future applications of PCR, when possible, should be aimed at constructing multiple detection systems in which a number of clinically and epidemiologically relevant pathogens (viruses, bacteria, parasites, and/or fungi) may be detected, characterized, and/or inevitably uncovered in a symptom- and/or system-specific manner.

REFERENCES

1. **Adleberg, J. M., and C. Wittwer.** 1995. Use of the polymerase chain reaction in the diagnosis of ocular disease. Curr. Opin. Ophthalmol. 6:80–85.
2. **Bakaletz, L. O., G. J. White, J. C. Post, and G. D. Ehrlich.** 1998. Blinded multiplex PCR analyses of middle ear and nasopharyngeal fluid from chinchilla models of single- and mixed-pathogen-induced otitis media. Clin. Diagn. Lab. Immunol. 5:219–224.
3. **Beards, G., C. Graham, and D. Pillay.** 1998. Investigation of vesicular rashes for HSV and HZV by PCR. J. Med. Virol. 54:155–157.
4. **Bej, A. K., M. H. Mahbubani, and R. M. Atlas.** 1991. Amplification of nucleic acids by polymerase chain reaction (PCR) and other methods and applications. Crit. Rev. Biochem. Mol. Biol. 26:301–334.
5. **Belli, A., B. Rodriguez, H. Aviles, and E. Harris.** 1998. Simplified polymerase chain reaction detection of new world *Leishmania* in clinical specimens of cutaneous leishmaniasis. Am. J. Trop. Med. Hyg. 58:102–109.
6. **Bertram, S., F. T. Hufert, J. van Lunzen, and D. von Laer.** 1996. Coinfection of individual leukocytes with human cytomegalovirus and human immunodeficiency virus is a rare event *in vivo*. J. Med. Virol. 49:283–288.
7. **Beyrer, C., K. Jitwatcharanan, C. Natpratan, R. Kaewvichit, K. E. Nelson, C. Y. Chen, J. B. Weiss, and S. A. Morse.** 1998. Molecular methods for the diagnosis of genital ulcer disease in a sexually transmitted disease clinic population in Northern Thailand: predominance of the herpes simplex virus infection. J. Infect. Dis. 178:243–246.
8. **Birch, D. E., L. Kolmodin, J. Wong, G. A. Zangenberg, M. A. Zoccoli, N. McKinney, and K. K. Y. Young.** 1996. Simplified hot start PCR. Nature 381:445–446.
9. **Brownie, J., S. Shawcross, J. Theaker, D. Whitcombe, R. Ferrie, C. Newton, and S. Little.** 1997. The elimination of primer-dimer accumulation in PCR. Nucleic Acids Res. 25:3235–3241.
10. **Buonagurio, D. A., J. W. Coleman, S. A. Patibandla, B. S. Prabhakar, and J. M. Tatem.** 1999. Direct detection of Sabin poliovirus vaccine strains in stool specimens of first-dose vaccinees by a sensitive reverse transcription-PCR method. J. Clin. Microbiol. 37:283–289.
11. **Caballero, O. L., C. L. P. Menezes, M. C. S. L. Costa, S. C. Fernandes, T. M. Anacleto, R. M. De Oliveira, E. A. Viotti, E. R. F. Tavora, S. S. Vilaca, E. Sabbaga, F. J. De Paula, P. F. Tavora, L. L. Villa, and A. J. G. Simpson.** 1997. Highly sensitive single-step PCR protocol for diagnosis and monitoring of human cytomegalovirus infection in renal transplant recipients. J. Clin. Microbiol. 35:3192–3197.
12. **Casas, I., A. Tenorio, J. M. Echevarria, P. E. Klapper, and G. M. Cleator.** 1997. Detection of enteroviral RNA and specific DNA of herpesviruses by multiplex genome amplification. J. Virol. Methods 66:39–50.
13. **Casas, I., F. Pozo, G. Trallero, J. M. Echevarria, and A. Tenorio.** 1999. Viral diagnosis of neurological infection by RT multiplex PCR: a search for entero- and herpesviruses in a prospective study. J. Med. Virol. 57:145–151.
14. **Cassinotti, P., and G. Siegl.** 1998. A nested-PCR assay for the simultaneous amplification of HSV-1, HSV-2, and HCMV genomes in patients with presumed herpetic CNS infection. J. Virol. Methods 71:105–114.
15. **Cassinotti, P., H. Mietz, and G. Siegl.** 1996. Suitability and clinical application of a multiplex nested PCR assay for the diagnosis of herpes simplex virus infections. J. Med. Virol. 50:75–81.
16. **Caudai, C., M. G. Padula, V. Bettini, and P. E. Valensin.** 1998. Detection of HCV and GBV-C/HGV infection by multiplex PCR in plasma samples of transfused subjects. J. Virol. Methods 70:79–83.
17. **Cha, R. S., and W. G. Thilly.** 1993. Specificity, efficiency, and fidelity of PCR. PCR Methods Appl. 3:S18–S29.
18. **Chamberlain, J. S., and J. R. Chamberlain.** 1994. Optimization of multiplex PCRs, p. 38–46. *In* K. B. Mullis, F. Ferre, and R. A. Gibbs (ed.), The polymerase chain reaction. Birkhauser, Boston, Mass.
19. **Chamberlain, J. S., R. A. Gibbs, J. E. Ranier, P. N. Nguyen, and C. T. Caskey.** 1989. Multiplex PCR for the diagnosis of Duchenne muscular dystrophy, p. 272–281. *In* D. H. Gelfand, M. A. Innis, J. J. Shinsky, and T. J. White (ed.), PCR protocols: a guide to methods and applications. Academic Press, San Diego, Calif.
20. **Chamberlain, J. S., R. A. Gibbs, J. E. Ranier, P. N. Nguyen, and C. T. Caskey.** 1988. Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification. Nucleic Acids Res. 16:11141–11156.
21. **Chou, Q., M. Russel, D. E. Birch, J. Raymond, and W. Bloch.** 1992. Prevention of pre-PCR mis-priming and primer dimerization improves low-copy-number amplifications. Nucleic Acids Res. 11:1717–1723.
22. **Cinque, P., L. Vago, R. Marenzi, B. Giudici, T. Weber, R. Corradini, D. Ceresa, A. Lazzarin, and A. Linde.** 1998. Herpes simplex virus infections of the central nervous system in human immunodeficiency virus-infected patients: clinical management by polymerase chain reaction assay of cerebrospinal fluid. Clin. Infect. Dis. 27:303–309.
23. **Clavel, C., S. Rihet, M. Masure, C. Chypre, J. C. Boulanger, C. Quereux, and P. Birembaut.** 1998. DNA-EIA to detect high and low risk HPV genotypes in cervical lesions with E6/E7 primer mediated multiplex PCR. J. Clin. Pathol. 51:38–43.
24. **Della, N. G.** 1996. Molecular biology in ophthalmology: a review of principles and recent advances. Arch. Ophthalmol. 114:457–463.
25. **DiCarlo, R. P., and D. H. Martin.** 1997. The clinical diagnosis of genital ulcer disease in men. Clin. Infect. Dis. 25:292–298.
26. **Dieffenbach, C. W., T. M. J. Lowe, and G. S. Dveksler.** 1993. General concepts for PCR primer design. PCR Methods Appl. 3:S30–S37.
27. **Echevarria, J. E., D. D. Erdman, E. M. Swierkosz, B. P. Holloway, and L. J. Anderson.** 1998. Simultaneous detection and identification of human parainfluenza viruses 1, 2, and 3 from clinical samples by multiplex PCR. J. Clin. Microbiol. 36:1388–1391.
28. **Edwards, M. C., and R. A. Gibbs.** 1994. Multiplex PCR: advantages, development, and applications. PCR Methods Appl. 3:S65–S75.
29. **Egger, D., L. Pasamontes, M. Ostermayer, and K. Bienz.** 1995. Reverse transcription multiplex PCR for differentiation between polio and enteroviruses from clinical and environmental samples. J. Clin. Microbiol. 33: 1442–1447.
30. **Ellis, L., D. M. Fleming, and M. C. Zambon.** 1997. Multiplex reverse transcription-PCR for surveillance of influenza A and B viruses in England and Wales in 1995 and 1996. J. Clin. Microbiol. 35:2076–2082.
31. **Elnifro, E. M., R. J. Cooper, P. E. Klapper, A. S. Bailey, and A. B. Tullo.** 1999. Diagnosis of viral chlamydial keratoconjunctivitis: which laboratory test? Br. J. Ophthalmol. 83:622–627.
32. **Emmanuel, P. J.** 1993. Polymerase chain reaction from bench to bedside.

Applications for infectious disease. J. Fla. Med. Assoc. 80:627–630.
33. Fan, J., K. J. Henrickson, and L. L. Savatski. 1998. Rapid simultaneous diagnosis of infections with respiratory syncytial viruses A and B, influenza viruses A and B, and human parainfluenza virus types 1, 2, and 3 by multiplex quantitative reverse transcription-polymerase chain reaction enzyme hybridization assay (Hexaplex). Clin. Infect. Dis. 26:1397–1402.
34. Garcia, L., J. C. Tercero, B. Legido, J. A. Ramos, J. Alemany, and M. Sanz. 1998. Rapid detection of *Actinobacillus actinomycetemcomitans*, *Prevotella intermedia* and *Porphyromona gingivalis* by multiplex PCR. J. Periodontal Res. 33:59–64.
35. Godfroid, E., M. Heinderyckx, F. Mansy, I. Fayt, J. C. Noel, L. Thiry, and A. Bollen. 1998. Detection and identification of human papilloma viral DNA, types 16, 18, and 33 by a combination of polymerase chain reaction and a colorimetric solid phase capture hybridisation assay. J. Virol. Methods 75:69–81.
36. Grondahl, B., W. Puppe, A. Hoppe, I. Khne, J. A. L. Weigl, and H. J. Schmitt. 1999. Rapid identification of nine microorganisms causing acute respiratory tract infection by single-tube multiplex reverse transcription-PCR: feasibility study. J. Clin. Microbiol. 37:1–7.
37. Harmon, K. M., G. M. Ransom, and I. V. Wesley. 1997. Differentiation of *Campylobacter jejuni* and *Campylobacter coli* by polymerase chain reaction. Mol. Cell. Probes 11:195–200.
38. Harris, E. 1996. Appropriate transfer of molecular technology. Asia Pacific Technol. Monitor 13:29–33.
39. Harris, E., G. Kropp, A. Belli, B. Rodriguez, and N. Agabian. 1998. Single-step multiplex PCR assay for characterization of New World *Leishmania* complexes. J. Clin. Microbiol. 36:1989–1995.
40. Harrison, T. J. 1998. The polymerase chain reaction—a time of transition from research to routine. J. Clin. Pathol. 51:491–492.
41. Hassan-Walker, A. F., A. V. Cope, P. D. Griffiths, and V. C. Emery. 1998. Transcription of the human cytomegalovirus natural killer decoy gene, UL18, *in vitro* and *in vivo*. J. Gen. Virol. 79:2113–2116.
42. Hendolin, P. H., A. Markkanen, J. Ylikoski, and J. J. Wahlfors. 1997. Use of multiplex PCR for simultaneous detection of four bacterial species in middle ear effusions. J. Clin. Microbiol. 35:2854–2858.
43. Henegariu, O., N. A. Heerema, S. R. Dlouhy, G. H. Vance, and P. H. Vogt. 1997. Multiplex PCR: critical parameters and step-by-step protocol. BioTechniques 23:504–511.
44. Hengen, P. N. 1997. Optimizing multiplex and LA-PCR with betaine. Trends Biol. Sci. 22:225–226.
45. Heredia, A., V. Soriano, S. H. Weiss, R. Bravo, A. Vallejo, T. N. Denny, J. S. Epstein, and I. K. Hewlett. 1996. Development of a multiplex PCR assay for the simultaneous detection and discrimination of HIV-1, HIV-2, HTLV-I and HTLV-II. Clin. Diagn. Virol. 7:85–92.
46. Hewlett, I. K., M. Ruta, K. Cristiano, C. A. Hawthorne, and J. S. Epstein. 1989. Co-amplification of multiple regions of the HIV-1 genome by the polymerase chain reaction: potential use in multiple diagnosis. Oncogene 4:1149.
47. Hu, K. O., C. H. Yu, S. Lee, F. G. Villamil, and J. M. Vierling. 1995. Simultaneous detection of both hepatitis B virus DNA and hepatitis C virus RNA using a combined one-step polymerase chain reaction technique. Hepatology 21:901–907.
48. Jackson, J. B. 1990. Polymerase chain reaction in transfusion medicine. Transfusion 30:51.
49. Jackson, R., D. J. Morris, R. J. Cooper, A. S. Bailey, P. E. Klapper, and G. M. Cleator. 1996. Multiplex polymerase chain reaction for adenovirus and herpes simplex virus in eye swabs. J. Virol. Methods 56:41–48.
50. Jacobi, V., G. D. Bachand, R. C. Hamelin, and J. D. Castello. 1998. Development of a multiplex immunocapture RT-PCR assay for detection and differentiation of tomato and tobacco mosaic tobamoviruses. J. Virol. Methods 74:167–178.
51. Jin, L., A. Richards, and D. W. G. Brown. 1996. Development of a dual target-PCR for detection and characterization of measles virus in clinical specimens. Mol. Cell. Probes 10:191–200.
52. Kaucner, C., and T. Stinear. 1998. Sensitive and rapid detection of viable *Giardia* cysts and *Cryptosporidium parvum* oocysts in large-volume water samples with wound fiberglass cartridge filters and reverse transcription-PCR. App. Environ. Microbiol. 64:1743–1749.
53. Kebelmann-Betzing, C., K. Seeger, S. Dragon, G. Schmitt, A. Moricke, T. A. Schild, G. Henze, and B. Beyermann. 1998. Advantages of a new *Taq* DNA polymerase in multiplex PCR and time-release PCR. BioTechniques 24: 154–158.
54. Klapper, P. E., D. L. Jungkind, T. Fenner, R. Antinozzi, J. Schirm, and C. Blanckmeister. 1998. Multicenter international work flow study of an automated polymerase chain reaction instrument. Clin. Chem. 44:1737–1739.
55. Kwok, S., and R. Higuchi. 1989. Avoiding false positives with PCR. Nature 339:237–238.
56. Lazarus, P., and S. Caruana. 1996. Typing of common human papilloma virus strains by multiplex PCR. Anal. Biochem. 243:198–201.
57. Linz, U., U. Delling, and H. Rübsamen-Waigmann. 1990. Systematic studies on parameters influencing the performance of the polymerase chain reaction. J. Clin. Chem. Clin. Biochem. 28:5–13.
58. Livache, T., H. Bazin, and G. Mathis. 1998. Conducting polymers on microelectronic devices as tools for biological analyses. Clin. Chim. Acta 278:171–176.
59. Mahony, J. B., D. Jang, S. Chong, K. E. Luinstra, J. W. Sellors, M. Tyndall, and M. Chernesky. 1997. Detection of *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Ureaplasma urealyticum*, and *Mycoplasma genitalium* in first-void urine specimens by multiplex polymerase chain reaction. Mol. Diagn. 2:161–168.
60. Mahony, J. B., K. E. Luinstra, M. Tyndall, J. W. Sellors, J. Krepel, and M. Chernesky. 1995. Multiplex PCR for detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in genitourinary specimens. J. Clin. Microbiol. 33:3049–3053.
61. Markoulatos, P., V. Samara, N. Siafakas, E. Plakokefalos, N. Spyrou, and M. L. Moncany. 1999. Development of a quadriplex polymerase chain reaction for human cytomegalovirus detection. J. Clin. Lab. Anal. 13:99–105.
62. McElhinney, L. M., R. J. Cooper, and D. J. Morris. 1995. Multiplex polymerase chain reaction for human herpesvirus-6, human cytomegalovirus, and human β-globin DNA. J. Virol. Methods 53:223–233.
63. Minjolle, S., C. Michelet, I. Jusselin, M. Joannes, F. Cartier, and R. Collimon. 1999. Amplification of the six major human herpesviruses from cerebrospinal fluid by a single PCR. J. Clin. Microbiol. 37:950–953.
64. Mitrani-Rosenbaum, S., R. Tsvieli, O. Lavie, R. Boldes, E. Anteby, S. Shimonovitch, T. Lazarovitch, and A. Friedmann. 1994. Simultaneous detection of three common sexually transmitted agents by polymerase chain reaction. Am. J. Obstet. Gynecol. 171:784–790.
65. Mitsuhashi, M. 1996. Technical report: part 2. Basic requirements for designing optimal PCR primers. J. Clin. Lab. Anal. 10:285–293.
66. Morse, S. A., D. L. Trees, Y. Htun, F. Radebe, K. A. Orle, Y. Dangor, C. M. Beck-Sague, S. Schmid, G. Fehler, J. B. Weiss, and R. C. Ballard. 1997. Comparison of clinical diagnosis and standard laboratory and molecular methods for the diagnosis of genital ulcer disease in Lesotho: association with human immunodeficiency virus infection. J. Infect. Dis. 175:583–589.
67. Moschettini, D., A. De Milito, M. Catucci, A. Marconi, C. Rinina, M. L. Bianchi-Bandinelli, and P. E. Valensin. 1998. Detection of human herpesvirus 6 and 7 in heart transplant recipients by multiplex polymerase chain reaction method. Eur. J. Clin. Microbiol. Infect. Dis. 17:117–119.
68. Mutter, G. L., and K. A. Boynton. 1995. PCR bias in amplification of androgen receptor alleles, a trinucleotide repeat marker used in clonality studies. Nucleic Acids Res. 23:1411–1418.
69. Myerson, D., P. A. Lingenfelter, C. A. Gleaves, J. D. Meyers, and R. A. Bowden. 1993. Diagnosis of cytomegalovirus pneumonia by polymerase chain reaction with archived frozen lung tissue and bronchoalveolar lavage fluid. Am. J. Clin. Pathol. 100:407–413.
70. Nadin-Davis, S. A., W. Huang, and A. I. Wandeler. 1996. The design of strain-specific polymerase chain reaction for discrimination of the raccoon rabies virus strain from indigenous rabies viruses of Ontario. J. Virol. Methods 57:1–14.
71. Nedjar, S., F. Mitchell, and R. Biswas. 1994. Simultaneous amplification and detection of specific hepatitis B virus and hepatitis C virus genomic sequences in serum samples. J. Med. Virol. 42:212–216.
72. Nicodème, P., and J. Steyaert. 1997. Selecting optimal oligonucleotide primers for multiplex PCR. Intelligent Syst. Mol. Biol. 51:210–213.
73. Orle, K. A., C. A. Gates, D. H. Martin, B. A. Body, and J. B. Weiss. 1996. Simultaneous PCR detection of *Haemophilus ducreyi*, *Treponema pallidum*, and herpes simplex virus types 1 and 2 from genital ulcers. J. Clin. Microbiol. 34:49–54.
74. Osiowy, C. 1998. Direct detection of respiratory syncytial virus, parainfluenza virus, and adenovirus in clinical respiratory specimens by a multiplex reverse transcription-PCR assay. J. Clin. Microbiol. 36:3149–3154.
75. Pizzighella, S., G. Pisoni, F. Bevilacqua, and A. Vaona. 1995. Simultaneous polymerase chain reaction detection and restriction typing for the diagnosis of human genital papillomavirus infection. J. Virol. Methods 55:245–256.
76. Polz, M. F., and C. M. Cavanaugh. 1998. Bias in template-to-product ratios in multitemplate PCR. Appl. Environ. Microbiol. 64:3724–3730.
77. Pozo, F., and A. Tenorio. 1999. Detection and typing of lymphotropic herpesviruses by multiplex polymerase chain reaction. J. Virol. Methods 79:9–19.
78. Pring-Akerblom, P., F. E. J. Trijssenaar, T. Adrian, and H. Hoyer. 1999. Multiplex polymerase chain reaction for subgenus-specific detection of human adenovirus in clinical samples. J. Med. Virol. 58:87–92.
79. Raeymaekers, L. 1995. A commentary on the practical applications of competitive PCR. Genome Res. 5:91–94.
80. Rajeev, B., and J. Biswas. 1998. Molecular biologic techniques in ophthalmic pathology. Indian J. Ophthalmol. 46:3–13.
81. Read, S. J., and J. B. Kurtz. 1999. Laboratory diagnosis of common viral infections of the central nervous system by using a single multiplex PCR screening assay. J. Clin. Microbiol. 37:1352–1355.
82. Read, S. J., K. J. M. Jeffery, and C. R. M. Bangham. 1997. Aseptic meningitis and encephalitis: the role of PCR in the diagnostic laboratory. J. Clin. Microbiol. 35:691–696.
83. Rees, W. A., T. D. Yager, J. Korte, and P. H. von Hippel. 1993. Betaine can

eliminate the base pair composition dependence of DNA melting. Biochemistry 32:137–144.
84. Repp, R., S. Rhiel, K. H. Heermann, S. Schaefer, C. Keller, P. Ndumbe, F. Lampert, and W. H. Gerlich. 1993. Genotyping by multiplex polymerase chain reaction for detection of endemic hepatitis B virus transmission. J. Clin. Microbiol. 31:1095–1102.
85. Reubel, G. H., B. S. Crabb, and M. J. Studdert. 1995. Diagnosis of equine gammaherpesvirus 2 and 5 infections by polymerase chain reaction. Arch. Virol. 140:1049–1060.
86. Rithidech, K. N., J. J. Dunn, and C. R. Gordon. 1997. Combining multiplex and touchdown PCR to screen murine microsatellite polymorphisms. BioTechniques 23:36–45.
87. Roberts, T. C., and G. A. Storch. 1997. Multiplex PCR for diagnosis of AIDS-related central nervous system lymphoma and toxoplasmosis. J. Clin. Microbiol. 35:268–269.
88. Robertson, J. M., and J. Walsh-Weller. 1998. An introduction to PCR primer design and optimisation of amplification reactions. Methods Mol. Biol. 98:121–154.
89. Rochelle, P. A., R. D. Leon, M. H. Stewart, and R. L. Wolfe. 1997. Comparison of primers and optimization of PCR conditions for detection of *Cryptosporidium parvum* and *Giardia lamblia* in water. J. Clin. Microbiol. 63:106–114.
90. Rodriguez, L. L., J. H. Owens, C. J. Peters, and S. T. Nichol. 1998. Genetic reassortment among viruses causing hantavirus pulmonary syndrome. Virology 242:99–106.
91. Roux, K. H. 1995. Optimization and troubleshooting in PCR. PCR Methods Appl. 4:S185–S194.
92. Ruano, G., D. E. Brash, and K. K. Kidd. 1991. PCR: the first few cycles. Amplifications 7:1–4.
93. Sevall, J. S. 1990. Detection of parvovirus B19 by dot-blot and polymerase chain reaction. Mol. Cell. Probes 4:237–246.
94. Sherlock, J., V. Cirigliano, M. Petrou, B. Tutschek, and M. Adinolfi. 1998. Assessment of diagnostic quantitative fluorescent multiplex polymerase chain reaction assays performed on single cells. Ann. Hum. Genet. 62:9–23.
95. Shuber, A. P., V. J. Grondin, and K. W. Klinger. 1995. A simplified procedure for developing multiplex PCRs. Genome Res. 5:488–493.
96. Shuber, A. P., J. Skoletsky, R. Stern, and B. L. Handelin. 1993. Efficient 12-mutation testing in the CFTR gene: a general model for complex mutation analysis. Hum. Mol. Genet. 2:153–158.
97. Soler, C., P. Allibert, Y. Chardonnet, P. Cros, B. Mandrand, and J. Thivolet. 1991. Detection of human papillomavirus types 6, 11, 16 and 18 in mucosal and cutaneous lesions by the multiplex polymerase chain reaction. J. Virol. Methods 35:143–157.
98. Stockton, J., J. S. Ellis, M. Saville, J. P. Clewley, and M. C. Zambon. 1998. Multiplex PCR for typing and subtyping influenza and respiratory syncytial viruses. J. Clin. Microbiol. 36:2990–2995.
99. Sunzeri, F. J., T. H. Lee, R. G. Bronlee, and M. P. Busch. 1991. Rapid simultaneous detection of multiple retroviral DNA sequences using the polymerase chain reaction and capillary DNA chromatography. Blood 77: 879–886.
100. Suzuki, M. T., and S. J. Giovannoni. 1996. Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. App. Environ. Microbiol. 62:625–630.
101. Taylor, G. R., and P. Robinson. 1998. The polymerase chain reaction: from functional genomics to high-school practical classes. Curr. Opin. Biotechnol. 9:35–42.
102. Tenorio, A., J. E. Echevarria, I. Casas, J. M. Echevarria, and E. Tabares. 1993. Detection and typing of human herpesviruses by multiplex polymerase chain reaction. J. Virol. Methods 44:261–269.
103. Vandenvelde, C., M. Verstraete, and D. Van Beers. 1990. Fast multiplex polymerase chain reaction on boiled clinical samples for rapid viral diagnosis. J. Virol. Methods 30:215–227.
104. Vangrysperre, W., and K. De Clercq. 1996. Rapid and sensitive polymerase chain reaction based detection and typing of foot-and-mouth disease virus in clinical samples and cell culture isolates, combined with a simultaneous differentiation with other genomically and/or symptomatically related viruses. Arch. Virol. 141:331–344.
105. Vesy, C. J., J. K. Greenson, A. C. Papp, P. J. Snyder, S. J. Qualman, and T. W. Prior. 1993. Evaluation of celiac disease biopsies for adenovirus 12 and using a multiplex polymerase chain reaction. Mod. Pathol. 6:61–64.
106. Vet, J. A. M., A. R. Majithia, S. A. E. Earras, S. Tyagi, S. Dube, B. J. Poiesz, and F. R. Kramer. 1999. Multiplex detection of four pathogenic retroviruses using molecular beacons. Proc. Natl. Acad. Sci. USA 96:6394–6399.
107. Wagar, E. A. 1996. Direct hybridization and amplification applications for the diagnosis of infectious diseases. J. Clin. Lab. Anal. 10:312–325.
108. Wagner, A., N. Blackstone, P. Cartwright, M. Dick, B. Misof, P. Snow, G. P. Wagner, J. Bartels, M. Murtha, and J. Pendleton. 1994. Surveys of gene families using polymerase chain reaction: PCR selection and PCR drift. Syst. Biol. 43:250–261.
109. Walsh, P. S., H. A. Erlich, and R. Higuchi. 1992. Preferential PCR amplification of alleles: mechanisms and solutions. PCR Methods Appl. 1:241–250.
110. Waters, L. C., S. C. Jacobson, N. Kroutchinina, J. Khandurina, R. S. Foote, and J. M. Ramsey. 1998. Microchip device for cell lysis, multiplex PCR amplification, and electrophoretic sizing. Anal. Chem. 70:158–162.
111. Wattel, E., M. Mariotti, F. Agis, E. Gordien, O. Prou, A. M. Courouce, P. Rouger, S. Wain-Hobson, I. S. Chen, and J. J. Lefrere. 1992. Human T lymphotropic virus (HTLV) type I and II DNA amplification in HTLV-I/II-seropositive blood donors of the French West Indies. J. Infect. Dis. 165:369–372.
112. Williams, J. F. 1989. Optimization strategies for the polymerase chain reaction. BioTechniques 7:762–769.
113. Wilson, I. G. 1997. Inhibition and facilitation of nucleic acid amplification. App. Environ. Microbiol. 63:3741–3751.
114. Wilson, W. C., and C. C. L. Chase. 1993. Nested and multiplex polymerase chain reactions for the identification of bluetongue virus infection in the biting midge, *Culicoides variipennis*. J. Virol. Methods 45:39–47.
115. Winn-Deen, E. S. 1996. Automation of molecular genetic methods. Part 2: DNA amplification techniques. J. Clin. Ligand Assay 19:21–26.
116. Wirz, B., J. D. Tratschin, H. K. Muller, and D. B. Mitchell. 1993. Detection of hog cholera and differentiation from other pestiviruses by polymerase chain reaction. J. Clin. Microbiol. 31:1148–1154.
117. Wolcott, M. J. 1992. Advances in nucleic acid-based detection methods. Clin. Microbiol. Rev. 5:370–386.
118. Wong, K. C., B. S. W. Ho, S. I. Egglestone, and W. H. P. Lewis. 1995. Duplex PCR system for simultaneous detection of *Neisseria gonorrhoeae* and *Chlamydia trachomatis* in clinical specimens. J. Clin. Pathol. 48:101–104.
119. Woo, P. C. Y., S. S. Chiu, W. Seto, and M. Peiris. 1997. Cost-effectiveness of rapid diagnosis of viral respiratory tract infections in pediatric patients. J. Clin. Microbiol. 35:1579–1581.
120. Wu, D. Y., L. Ugozzoli, B. K. Pal, J. Qian, and R. B. Wallace. 1991. The effect of temperature and oligonucleotide primer length on the specificity and efficiency of amplification by the polymerase chain reaction. DNA Cell. Biol. 10:233–238.
121. Yang, C.-F., L. De, B. P. Holloway, M. A. Pallansch, and O. M. Kew. 1991. Detection and identification of vaccine-related polioviruses by polymerase chain reaction. Virus Res. 20:159–179.
122. Yourno, J. 1992. A method for nested PCR with single closed reaction tubes. PCR Methods Appl. 2:60–65.
123. Zheng, P. S., S. R. Li, T. Iwasaka, J. Song, M. H. Gui, and H. Sugimori. 1995. Simultaneous detection by consensus multiplex PCR of high- and low-risk and other types of human papilloma virus in clinical samples. Gynecol. Oncol. 58:179–183.
124. Zimmermann, K., D. Schogl, B. Plaimauer, and J. W. Mannhalter. 1996. Quantitative multiple competitive PCR of HIV-1 DNA in a single reaction tube. BioTechniques 21:480–484.
125. Zou, S. 1997. A practical approach to genetic screening for influenza virus variants. J. Clin. Microbiol. 35:2623–2627.
126. Zou, S., C. Stansfield, and J. Bridge. 1998. Identification of new influenza B virus variants by multiplex reverse transcription-PCR and the heteroduplex mobility assay. J. Clin. Microbiol. 36:1544–1548.

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Inhibition of real-time RT–PCR quantification due to tissue-specific contaminants

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Abstract

Real-time reverse transcription–polymerase chain reaction (RT–PCR) is currently considered the most sensitive method to study low abundance gene expression. Since comparison of gene expression levels in various tissues is often the purpose of an experiment, we studied a tissue-linked effect on nucleic acid amplification. Based on the raw data generated by a LightCycler instrument, we propose a descriptive mathematical model of PCR amplification. This model allowed us to study amplification kinetics of four common housekeeping genes in total RNA samples derived from various bovine tissues. We observed that unknown tissue-specific factors can influence amplification kinetics but this affect can be ameliorated, in part, by appropriate primer selection.

Keywords: Quantitative polymerase chain reaction; Real-time reverse transcription–polymerase chain reaction; Gene expression; Housekeeping genes; Ubiquitin; β-actin; GAPDH; 18S rRNA

1. Introduction

Reverse transcription–polymerase chain reaction (RT–PCR) is the method of choice for quantifying low abundant mRNAs in material such as cells and tissues [1–4]. This method is fast and highly reproducible. Further, its high sensitivity is its principal advantage over other techniques.

In real-time PCR the quantification takes place within an exponential phase of the amplification curve [5]. A crossing point (CP) or threshold cycle (Ct) is then extrapolated to determine a starting amount of template molecules. The CP gives the researcher the first raw information about the expression level of a given gene.

All methods of gene quantification report their findings relative to a measurable base (e.g. copies per cell, weight of tissue, volume of blood, etc.). The correct choice of the denominator depends on the question asked and can significantly affect the quality of the results [6]. To obtain an actual number of copies, various 'absolute' standards are often employed [7–9], but even in these cases, the quantification is always relative as some errors in a protocol are inevitably present [6,10]. So called housekeeping or maintenance genes [11] such as actins, tubulins, albumins, ubiquitin, glyceraldehyd-3-phosphate dehydrogenase (GAPDH), 18S or 28S ribosomal subunits (rRNA) are often used as relative standards [12]. These genes are believed to undergo little, if any, variation in expression under most experimental treatments. Yet, there have been many reports on the regulation of these genes [12–14].

Another important criterion for reliable measurement and comparison of more than one gene is that all of the genes amplify equally. Experiments using normalization with housekeeping genes often overlook this parameter despite the fact that corrections have already be suggested in the literature [15–19].

Many factors present in samples as well as exogenous contaminants have been shown to inhibit PCR (review in Refs. [20,21]). For example, the presence of hemoglobin, fat, glycogen, cell constituents, Ca^{2+}, DNA or RNA concentration, and DNA binding proteins are important factors [20,21]. Additionally, exogenous contaminants such as glove powder and phenolic compounds from

Abbreviations: RT–PCR, reverse transcription–polymerase chain reaction; CP, crossing point; GAPDH, glyceraldehyd-3-phosphate dehydrogenase; FDM, first derivative maximum; SDM, second derivative maximum.

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the extraction process or the plastic ware can have an inhibiting effect [20,21].

Since some experiments compare gene expression in different organs [9,22], tissue-specific inhibition of DNA amplification may be important. To study the amplification inhibition associated with three randomly chosen tissue types we proposed a mathematical model describing the DNA amplification kinetics in real-time PCR. Using this model we could compare parameters of the amplification kinetics and analyze them statistically.

2. Materials and methods

2.1. Preparation of cDNA samples

Samples of cerebellum, muscle and liver were gathered from six slaughtered cows, immediately frozen in liquid nitrogen and then stored at −80 °C until the total RNA extraction procedure was performed.

Tissue samples were homogenized and total RNA was extracted with a commercially available product, peqGOLD TriFast (Peqlab, Erlangen, Germany), utilizing a single modified liquid separation procedure [23]. No additional purification was performed. Constant amounts of 1000 ng of RNA were reverse-transcribed to cDNA using 200 units of MMLV Reverse Transcriptase (Promega, Mannheim, Germany) according to the manufacturers instructions.

Integrity of the DNA was determined by electrophoresis on 1% agarose gels. Nucleic acid concentrations were measured on a spectrophotometer (BioPhotometer, Eppendorf, Hamburg, Germany) at $OD_{260\ nm}$ with 220–1600 nm UVettes (Eppendorf). Purity of the RNA extracted was determined as the $OD_{260\ nm}/OD_{280\ nm}$ ratio with expected values between 1.8 and 2.0 (BioPhotometer). A possible trend between the samples and their $OD_{260\ nm}/OD_{280\ nm}$ values was examined.

2.2. Real-time PCR fluorescence data acquisition

Primer sequences of four common housekeeping genes; ubiquitin, β-actin, GAPDH and 18S rRNA were designed to span at least one intron (except for 18S rRNA) and synthesized commercially (MWG Biotech, Ebersberg, Germany) as shown in Table 1. PCR conditions were optimized on a gradient cycler (T-Gradient, Biometra, Göttingen, Germany) and subsequently on a LightCycler (Roche Diagnostic, Mannheim, Germany) [24] by analyzing the melting curves of the products [25]. Real-time PCR using SYBR Green I technology [26] on the LightCycler was then carried out to amplify cDNAs from the tissue samples.

Master-mix for each PCR run was prepared as follows: 6.4 μl of water, 1.2 μl $MgCl_2$ (4 mM), 0.2 μl of each primer (4 pmol), 1.0 μl Fast Start DNA Master SYBR Green I mix (Roche Diagnostics). Finally, 9 μl of master-mix and 25 ng of reverse transcribed total RNA in 1 μl water were transferred into capillaries (end volume 10 μl).

The following amplification program was used: After 10 min of denaturation at 95 °C, 40 cycles of real-time PCR with three-segment amplification were performed with: 15 s at 95 °C for denaturation, 10 s at respective annealing temperature (Table 1) and 20 s at 72 °C for elongation. A melting step was then performed with slow heating starting at 60 °C with a rate of 0.1 °C/s up to 99 °C with continuous measurement of fluorescence. The same gene was always quantified in each run to prevent any inter-run variation.

Fluorescence data from real-time PCR experiments were taken directly from LightCycler software version 3 (Roche Diagnostics), exported to SigmaPlot 2000 (SPSS, Munich, Germany) and fitted with a '*Four-parametric sigmoid model*' as described earlier by our group [27]. Parameters a, b, x_0 and y_0 of each fit were documented together with the coefficient of determination r^2.

All statistics were done in SigmaPlot 2000 (SPSS) and SigmaStat 2.0 (SPSS, Jandel Corporation).

2.3. Crossing point (CP) acquisition

On each individual real-time PCR run, five different CPs were acquired based on different determination procedures. First, the CP was placed into the first derivative maximum ($FDM_{SM} = x_0$) and into the second derivative maximum of the four-parametric sigmoid model (SDM_{SM}) of each run as shown earlier [27].

Table 1
Details of primers used to amplify four housekeeping genes

Gene	Primers	Sequence length (bp)	Annealing temperature (°C)
Ubiquitin	for: AGA TTC AGG ATA AGG AAG GCA T rev: GCT CCA CCT CCA GGG TGA T	198	60
GAPDH	for: GTC TTC ACT ACC ATG GAG AAG G rev: TCA TGG ATG ACC TTG GCC AG	197	58
18S rRNA	for: GAG AAA CGG CTA CCA CAT CCA A rev: GAC ACT CAG CTA AGA GCA TCG A	338	60
β-actin	for: AAC TCC ATC ATG AAG TGT GAC G rev: GAT CCA CAT CTG CTG GAA GG	234	60

Table 2
Two-way ANOVA

Factor	a	b	FDM_{SM}	SDM_{SM}	FP_{LC}	SDM_{LC}	CP_{Tm}
Tissue	0.01	<0.001	0.004	0.02	0.005	0.008	0.004
Gene	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Tissue–gene interaction	0.004	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

P-values of significance. Each of three rows indicates either one of factors or their interaction. In columns, *P*-values of effect of factors (or interaction) on respective parameter are shown.

Further, CP was computed using the '*Fit point method*' (FP_{LC}) [5] and '*Second derivative maximum method*' (SDM_{LC}) [5,28], both part of the LightCycler software 3.3 (Roche Diagnostics). In the FP_{LC} method, uninformative background fluorescence observations were discarded by setting a constant noise band. An intersecting line was then arbitrarily placed at the base of the exponential portion of the amplification curves. This generated CPs acquired at a constant fluorescence level (value 2 in our case).

In the SDM_{LC} method the second derivative maximum is calculated by LightCycler software based on an unknown and unpublished mathematical approximation of partial amplification kinetics around the supposed SDM_{LC} [5,28]. The FP_{LC} and SDM_{LC} were directly obtained from the calculated values by the LightCycler software 3.3 (Roche Diagnostics).

Eventually, the '*Taqman threshold level*' (Ct) or CP [29] computing method was simulated by fitting the intersecting line upon the 10 times value of ground fluorescence standard deviation (CP_{Tm}). In the '*Taqman threshold level*' procedure, the y_0 values of the four-parametric sigmoid model were considered ground fluorescence.

While parameters a and b describe amplification kinetics, FDM_{SM}, SDM_{SM}, FP_{LC}, SDM_{LC}, and CP_{Tm} are considered quantification parameters since they are clearly defined constants within the model.

Table 3a
Statistically processed parameters a, b, FDM_{SM}, SDM_{SM}, FP_{LC}, SDM_{LC}, CP_{Tm}, and r^2 of ubiquitin amplification

Tissue		a	b	FDM_{SM}	SDM_{SM}	FP_{LC}	SDM_{LC}	CP_{Tm}	r^2
Cerebellum	Mean	43.118	1.950	25.649	23.082	20.180	21.817	22.680	1.000
	CV (%)	9.56	1.17	1.31	1.50	2.13	1.43	1.77	0.004
Liver	Mean	39.355	2.004	26.184	23.545	20.688	22.288	22.597	1.000
	CV (%)	7.79	1.62	1.43	1.64	1.73	1.79	1.47	0.010
Muscle	Mean	41.958	2.064	26.443	23.725	20.637	22.487	25.370	0.999
	CV (%)	5.40	2.25	0.81	0.94	1.52	1.15	0.67	0.018
$Mean_{total}$		41.477	2.006	26.092	23.450	20.502	22.197	23.549	1.000
$CV_{in\text{-}tissue}$ (%)		7.58	1.68	1.18	1.36	1.79	1.46	1.30	0.011
$CV_{out\text{-}tissue}$ (%)		4.65	2.85	1.55	1.41	1.36	1.55	6.70	0.014

P-values of significance. Each of three rows indicates either one of factors or their interaction. In columns, *P*-values of effect of factors (or interaction) on respective parameter are shown.

Table 3b
Statistically processed parameters a, b, FDM_{SM}, SDM_{SM}, FP_{LC}, SDM_{LC}, CP_{Tm}, and r^2 of GAPDH amplification

Tissue		a	b	FDM_{SM}	SDM_{SM}	FP_{LC}	SDM_{LC}	CP_{Tm}	r^2
Cerebellum	Mean	47.223	2.075	23.663	20.930	18.185	19.583	20.483	0.998
	CV (%)	11.48	1.43	1.14	1.36	1.90	1.43	2.01	0.009
Liver	Mean	46.675	2.094	24.936	22.179	19.322	20.868	21.580	0.998
	CV (%)	6.39	2.75	1.61	1.97	2.09	2.21	2.20	0.020
Muscle	Mean	52.415	2.228	21.588	18.653	15.800	17.440	16.377	0.997
	CV (%)	3.79	2.94	3.30	4.08	4.70	4.06	4.48	0.032
$Mean_{total}$		48.771	2.132	23.396	20.587	17.769	19.297	19.480	0.998
$CV_{in\text{-}tissue}$ (%)		7.22	2.37	2.02	2.47	2.90	2.57	2.89	0.020
$CV_{out\text{-}tissue}$ (%)		6.50	3.92	7.22	8.68	10.12	8.98	14.08	0.068

P-values of significance. Each of three rows indicates either one of factors or their interaction. In columns, *P*-values of effect of factors (or interaction) on respective parameter are shown.

Table 3c
Statistically processed parameters a, b, FDM_{SM}, SDM_{SM}, FP_{LC}, SDM_{LC}, CP_{Tm}, and r^2 of 18S rRNA amplification

Tissue		a	b	FDM_{SM}	SDM_{SM}	FP_{LC}	SDM_{LC}	CP_{Tm}	r^2
Cerebellum	Mean	49.782	2.701	15.274	11.717	9.518	10.556	10.923	0.996
	CV (%)	3.76	5.33	3.64	6.26	6.32	5.83	6.17	0.047
Liver	Mean	53.544	2.897	14.669	10.854	8.638	9.809	9.185	0.996
	CV (%)	3.35	2.55	9.01	12.21	12.28	12.38	11.15	0.040
Muscle	Mean	55.943	2.752	15.369	11.744	9.250	10.573	10.267	0.997
	CV (%)	2.67	2.31	5.61	7.76	8.32	7.94	7.75	0.041
$Mean_{total}$		53.090	2.784	15.104	11.439	9.135	10.313	10.125	0.996
$CV_{in\text{-}tissue}$ (%)		3.26	3.40	6.09	8.74	8.97	8.72	8.36	0.042
$CV_{out\text{-}tissue}$ (%)		5.85	3.66	2.51	4.43	4.94	4.23	8.67	0.019

P-values of significance. Each of three rows indicates either one of factors or their interaction. In columns, P-values of effect of factors (or interaction) on respective parameter are shown.

2.4. Statistical evaluation of model parameters

Two-way ANOVA with tissue as the first factor of three levels (cerebellum, muscle and liver) and gene as the second factor of four levels (ubiquitin, β-actin, GAPDH, 18S rRNA) was applied to the parameters a, b, FDM_{SM}, SDM_{SM}, FP_{LC}, SDM_{LC} and CP_{Tm} (Table 2). Normal distribution was given within the data sets.

For all above-mentioned parameters and r^2 following statistical indicators were calculated (Tables 3a–3d)

- Interaction mean (i.e. from the six values within one level of factor gene and one level of factor tissue) and interaction coefficient of variance-CV.
- Total mean ($mean_{total}$) out of 18 values (always six samples in three tissues) for each factor gene.
- Mean value out of three CVs ($CV_{in\text{-}tissue}$) reporting internal variance within all three tissue levels.
- Coefficient of variance out of three interaction means ($CV_{out\text{-}tissue}$) showing a variability caused by factor tissue.

3. Results and discussion

All primers used could satisfactorily amplify the flanked sequence. The melting curve analysis and gel analysis detected very little, if any, nonspecific product. We approximated the PCR amplification kinetics with the four-parametric sigmoid model. This model describes well (in all data sets $r^2 > 0.99, n = 40$) the entire fluorescence curve and therefore its beginning and end do not need to be arbitrarily delimited [19]. Nevertheless, correlation between values of b and r^2 showed that there were differences in the goodness of the fit (Pearson correlation coefficient $r = 0.915, n = 72$). The best fit was in runs with high amplification efficiencies. With decreasing amplification efficiency the determination power of the model also decreased.

There is an integral purification step at the end of the extraction procedure [23], consisting of repeated washing the final total RNA pellet with ethanol. In this study no additional RNA purification was performed since additional purification decreases yield and is often omitted. This procedure simulated a routine PCR sample preparation as it is carried out in most labs. The contamination within the RNA samples detected as $OD_{260\ nm}/OD_{280\ nm}$ ratios was not significantly related to the type of tissue (data not shown).

Statistical analysis of the parameters a and b (Table 2) under an influence of the two experimental factors showed that the tissue was the largest source of variance and the primer sequences had the least affect [21,22].

A similar trend of variability within the log-linear trajectory slope (b) and plateau height (a) showed that the tissue from

Table 3d
Statistically processed parameters a, b, FDM_{SM}, SDM_{SM}, FP_{LC}, SDM_{LC}, CP_{Tm}, and r^2 of β-actin amplification

Tissue		a	b	FDM_{SM}	SDM_{SM}	FP_{LC}	SDM_{LC}	CP_{Tm}	r^2
Cerebellum	Mean	85.015	1.418	22.499	20.632	16.640	19.362	19.643	1.000
	CV (%)	5.11	2.15	2.22	2.54	3.21	2.69	2.34	0.004
Liver	Mean	86.694	1.467	23.555	21.624	17.400	20.348	18.633	1.000
	CV (%)	2.14	1.31	0.85	0.95	1.52	1.11	1.11	0.002
Muscle	Mean	84.886	1.470	24.264	22.328	18.230	21.047	20.813	1.000
	CV (%)	2.75	3.53	0.90	1.03	1.14	1.16	0.88	0.005
$Mean_{total}$		85.532	1.452	23.440	21.528	17.423	20.252	19.697	1.000
$CV_{in\text{-}tissue}$ (%)		1.00	2.33	1.32	1.51	1.96	1.65	1.45	0.004
$CV_{out\text{-}tissue}$ (%)		1.18	2.01	3.79	3.96	4.56	4.18	5.54	0.001

P-values of significance. Each of three rows indicates either one of factors or their interaction. In columns, P-values of effect of factors (or interaction) on respective parameter are shown.

which total RNA was extracted has a significant effect on the PCR kinetics and thus on the CP acquisition (Table 2). This can be caused by different amounts of cellular debris present in samples after RNA extraction [30,31]. Also endogenous contaminants such as blood or fat play an important role. Contamination of the sample may affect both the PCR as well as the preceding RT reaction [20,21].

Since interaction between both factors; tissue and gene is significant, the tissue-specific disturbance is not the same for all four amplified sequences but rather is sequence-specific. In our study, the highest resistance to tissue-specific disturbance showed the sequence of β-actin followed by ubiquitin, 18S rRNA and GAPDH (see $CV_{out\text{-}tissue}$ values in Tables 3a–3d). A plausible explanation of this interaction may be the presence of specific DNA blocking by polysaccharides or proteins present as endogenous contaminants in the sample [32]. It is possible that DNA amplification may be affected by regions of the template DNA that are specifically blocked by these endogenous macromolecules. Our data show that not only the choice of housekeeping genes [12–14] but also tissue-specific factors and the sequence-specific factors can affect the expression assays.

Tissue-specific suppression can be compensated, in part, by well performing primers such as those for β-actin and ubiquitin used here. From this data it seems that sequences that amplified with higher efficiency (i.e. small *b*) better resist inhibition and show lower variance in all parameters of the PCR kinetics (compare $mean_{total}$ of *b* and $CV_{out\text{-}tissue}$ values in Tables 3a and 3d with Tables 3b and 3c). Thus, primer selection and documenting the reaction efficiency are important PCR optimization steps. Although housekeeping genes are expressed differently in various tissues our data show that some vary less than others. For example, ubiquitin showed marginally higher variance between tissues than within one tissue (compare $CV_{out\text{-}group}$ with $CV_{in\text{-}group}$ in Table 3a). This suggests that the expression of ubiquitin in the different tissues was similar. The low variance for ubiquitin expression between tissues suggests that it is the best standard but is closely followed by β-actin and GAPDH. 18S rRNA, with its high variance, seems to be less suitable as an internal standard. This order was preserved in all CP computing methods.

Each method of computing CPs seems to be accurate for estimating expression levels but they varied slightly when CP acquisitions took place at different heights of the amplification curve (Tables 3a–3d). The method of first and second derivative maximum computed from the four-parametric sigmoid model is reliable and simple and generates reliable CPs comparable with other methods (see CV values in Tables 3a–3d).

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References

[1] Schmittgen TD. Real-time quantitative PCR. Methods 2001;25:383–5.
[2] Orlando C, Pinzani P, Pazzagli M. Developments in quantitative PCR. Clinical Chemistry and Laboratory Medicin 1998;36:255–69.
[3] Gibson UE, Heid CA, Williams PM. A novel method for real time quantitative RT–PCR. Genome Research 1996;6:995–1001.
[4] Freeman WM, Walker SJ, Vrana KE. Quantitative RT–PCR: pitfalls and potential. BioTechniques 1999;26:112–25.
[5] Rasmussen R. Quantification on the lightcycler instrument. In: Meuer S, Wittwer C, Nakagawara K, editors. Rapid cycle real-time PCR: methods and applications. Heidelberg: Springer; 2001. p. 21–34.
[6] Ferré F. Quantitative or semi-quantitative PCR: reality versus myth. PCR Methods and Applications 1992;2:1–9.
[7] Pfaffl MW, Hageleit M. Validities of mRNA quantification using recombinant RNA and recombinant DNA external calibration curves in real-time RT–CR. Biotechnology Letters 2001;23:275–82.
[8] Bustin SA. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. Journal of Molecular Endocrinology 2000;25:169–93.
[9] Tichopad A, Pfaffl MW, Didier A. Tissue-specific expression pattern of bovine prion: quantification using real-time RT–PCR. Molecular and Cellular Probes 2003;17:5–10.
[10] Souazé F, Ntodou-Thomé A, Tran CY, Rostene W, Forgez P. Quantitative RT–PCR: limits and accuracy. BioTechniques 1996;21:280–5.
[11] Warrington JA, Nair A, Mahadevappa M, Tsygantskaya M. Comparison of human adult and fetal expression and identification of 535 housekeeping/maintenance genes. Physiological Genomics 2000;2:143–7.
[12] Thellin O, Zorzi W, Lakaye B, De Borman B, Coumans B, Hennen G, Grisar T, Igout A, Heinen E. Housekeeping genes as internal standards: use and limits. Journal of Biotechnology 1999;75:291–5.
[13] Schmittgen TD, Zakrajsek BA. Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT–PCR. Journal of Biochemical and Biophysical Methods 2000;20:69–81.
[14] Suzuki T, Higgins PJ, Crawford DR. Control selection for RNA quantitation. BioTechniques 2000;29:332–7.
[15] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)). Methods 2001;25:402–8.
[16] Pfaffl MW. A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Research 2001;29(9):e45.
[17] Pfaffl MW, Horgan GW, Dempfle L. Relative Expression Software Tool (REST©) for group wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Research 2002;30(9):e36.
[18] Meijerink J, Mandigers C, van de Locht L, Tonnissen E, Goodsaid F, Raemaekers J. A novel method to compensate for different amplification efficiencies between patient DNA samples in quantitative real-time PCR. Journal of Molecular Diagnostics 2001;3:55–61.
[19] Weihong L, Saint A. A new quantitative method of real time reverse transcription polymerase chain reaction assay based on simulation of polymerase chain reaction kinetics. Analytical Biochemistry 2002;302:52–9.
[20] Wilson IG. Inhibition and facilitation of nucleic acid amplification. Applied and Environmental Microbiology 1997;63:3741–51.
[21] Rossen L, Norskov P, Holmstrom K, Rasmussen FO. Inhibition of PCR by components of food sample, microbial diagnostic assay and DNA-extraction solutions. International Journal of Food Microbiology 1992;17:37–45.
[22] Pfaffl MW, Lange IG, Daxenberger A, Meyer HH. Tissue-specific expression pattern of estrogen receptors (ER): quantification of ER

alpha and ER beta mRNA with real-time RT–PCR. Acta Pathologica Microbiologica et Immunologica Scandinavica 2001; 109:345–55.

[23] Chomczynski PA. Reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. BioTechniques 1993;15:532–4.

[24] Wittwer CT, Ririe KM, Andrew RV, David DA, Gundry RA, Balis UJ. The lightcycler: a microvolume multisample fluorimeter with rapid temperature control. BioTechniques 1997;22:176–81.

[25] Ririe KM, Rasmussen RT, Wittwer CT. Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. Analytical Biochemistry 1997;245:154–60.

[26] Morrison TB, Weis JJ, Wittwer CT. Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification. BioTechniques 1998;24:954–8.

[27] Tichopad A, Dzidic A, Pfaffl MW. Improving quantitative real-time RT–PCR reproducibility by boosting primer-linked amplification efficiency. Biotechnology Letters 2002;24:2053–6.

[28] Wittwer CT, Gutekunst M, Lohmann S. Method for quantification of an analyte. United States Patent No. US, 6,303,305 B1

[29] Holland PM, Abramson RD, Watson R, Gelfand DH. Detection of specific polymerase chain reaction product by utilizing the 5'–3' exonuclease activity of *Thermus aquaticus* DNA polymerase. Proceedings of the National Academy of Science of the United States of America 1991;15:7276–80.

[30] Wilson IG, Gilmour A, Cooper JE. Detection of toxigenic microorganisms in foods by PCR. In: Kroll RG, Gilmour A, Sussman M, editors. In new techniques in food and beverage microbiology. London: Blackwell; 1993. p. 163–72.

[31] Starnbach MN, Falkow S, Tomkins SL. Species-specific detection of *Legionella pneumophila* in water by DNA amplification and hybridization. Journal of Clinical Microbiology 1989;27:1257–61.

[32] Rijpens NP, Jannes G, Van Asbroeck M, Rossau R, Herman LMF. Direct detection of *Brucella* spp. In raw milk by PCR and reverse hybridization with 16S–23S rRNA spacer probes. Applied Environmental Microbiology 1996;62:1683–8.

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Minireview

Bioinformatic tools and guideline for PCR primer design

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Bioinformatics has become an essential tool not only for basic research but also for applied research in biotechnology and biomedical sciences. Optimal primer sequence and appropriate primer concentration are essential for maximal specificity and efficiency of PCR. A poorly designed primer can result in little or no product due to non-specific amplification and/or primer-dimer formation, which can become competitive enough to suppress product formation. There are several online tools devoted to serving molecular biologist design effective PCR primers. This review intends to provide a guide to choosing the most efficient way to design a new specific-primer by applying current publicly available links and Web services. Also, the purpose here is to provide general recommendations for the design and use of PCR primers.

Key words: Bio-computing, primer design, web-based resources.

INTRODUCTION

In the last 10 to 15 years the computer has become an essential companion for cell and molecular biologists. Bioinformatics is an emerging scientific discipline that uses information technology to organize, analyze, and distribute biological information in order to answer complex biological questions. Bioinformatics is an interdisciplinary research area, which may be broadly defined as the interface between biological and computational sciences (Singh and Kumar, 2001). It involves the solution of complex biological problems using computational tools and systems. It also includes the collection, organization, storage and retrieval of biological information from databases. Selection of oligonucleotide primers is useful for polymerase chain reaction (PCR), oligo hybridization and DNA sequencing. Proper primer design is actually one of the most important factors/steps in successful DNA sequencing. Various bioinformatics programs are available for selection of primer pairs from a template sequence. The plethora programs for PCR primer design reflects the central role of PCR in modem molecular biology. Nevertheless, all these computer programs are written mainly to assist in the primer design process and are not meant to replace the eye of the experienced researcher, especially considering the sometimes erratic nature of PCR experiments. When scheduling important PCR experiments, it is usually worthwhile to evaluate the predictions of numerous different programs and to use common sense and laboratory experience to evaluate the suggested primers before committing to their synthesis (Binas, 2000). This review summarizes the general guidelines for primer design online.

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WEB-BASED RESOURCES FOR PRIMER DESIGN

There are a numerous web-based resources for PCR and primer design. Though most are freely available, they are of variable quality and not well maintained. This often results in missing links and so sites that may have been useful previously may not be functional at a later date. There are a number of criteria that need to be established in the design of primers and a number of these are listed below (Tables 1and 2).

SOFTWARE IN PRIMER DESIGN

The use of software in biological applications has given a new dimension to the field of bioinformatics. Many different programs for the design of primers are now available. Freeware software is available on the internet and many universities have established servers where a user can log on and perform free analyses of proteins and nucleic acid sequences (Singh and Kumar, 2001). There are number of simple stand-alone programs as well as complex integrated networked versions of the commercial software available. These software packages may be for complete DNA and protein analysis, secondary structure predictions, primer design, molecular modeling, development of cloning strategies, plasmid drawing or restriction enzyme analyses. Companies engaged in biosoftware development include: Alkami Biosystems, Molecular Biology Insights, PREMIER Biosoft International, IntelliGenetics Inc., Hitachi Inc., DNA Star, Advanced American Biotechnology and Imaging. Some scientists have also developed algorithms and computer programs for various purposes of primer design (Rychlik and Rhoades, 1989; Lowe et al., 1990; Lucas et al., 1991; O'Hara and Venezia, 1991; Tamura et

Table 1. Online primer design sites.

Tool name	Description	www
CODEHOP	Consensus Degenerate Hybrid Oligonucleotide Primers; degenerate PCR primer design; will accept unaligned sequences.	http://blocks.fhcrc.org/codehop.html
Gene Fisher	Interactive primer design tool for standard or degenerate primers; will accept unaligned sequences.	http://bibiserv.techfak.uni-bielefeld.de/genefisher/
DoPrimer	Easily design primers for PCR and DNA sequencing.	http://doprimer.interactiva.de/
Primer3	Comprehensive PCR primer and hybridization probe design tool; many options but easy to accept defaults at first.	http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi http://www.basic.nwu.edu/biotools/Primer3.html http://www.justbio.com/primer/index.php
Primer Selection	Select PCR primers from nucleotide sequence.	http://alces.med.umn.edu/rawprimer.html
Web Primer	Allow alternative design of primers for either PCR or sequencing purpose.	http://genome-www2.stanford.edu/cgi-bin/SGD/web-primer
PCR Designer	For restriction analysis of sequence mutations.	http://cedar.genetics.soton.ac.uk/public_html/primer.html
Primo Pro 3.4	Reduces PCR noise by lowering the probability of random primering.	http://www.changbioscience.com/primo/primo.html
Primo Degenerate 3.4	Primo Degenerate 3.4 designs PCR primers based on a single peptide sequence or multiple alignments of proteins or nucleotides.	http://www.changbioscience.com/primo/primod.html
PCR Primer Design	An application that designs primers for PCR or sequencing purposes.	http://pga.mgh.harvard.edu/servlet/org.mgh.proteome.Primer
The Primer Generator	The program analyzes the original nucleotide sequence and desired amino acid sequence and designs a primer that either has a new restriction enzyme site or is missing an old one.	http://www.med.jhu.edu/medcenter/primer/primer.cgi
EPRIMER3	Picks PCR primers and hybridization oligos (EMBOSS).	http://bioweb.pasteur.fr/seqanal/interfaces/eprimer3.html
PRIMO	Prediction of forward and reverse oligonucleotide Primers.	http://bioweb.pasteur.fr/seqanal/interfaces/primo.html3 http://atlas.swmed.edu/primo/primo_form.html
PrimerQuest	A primer design tool.	http://www.idtdna.com/biotools/primer_quest/primer_quest.asp
MethPrimer	Design primers for methylation PCRs.	http://itsa.ucsf.edu/~urolab/methprimer/index1.html
Rawprimer	A tool for selection of PCR primers.	http://alces.med.umn.edu/rawprimer.html
MEDUSA	A tool for automatic selection and visual assessment of PCR primer pairs.	http://www.cgr.ki.se/cgr/MEDUSA/
The Primer Prim'er Project	Software suite that completely automates the PCR primer design process.	http://www-nmr.cabm.rutgers.edu/bioinformatics/Primer_Primer_Project/Primer.html
Oligonucleotides for the PCR	Seek oligonucleotides on both sides of an area.	http://www.citi2.fr/bio2/Oligo2lib.html
GAP	Genome- wide Automated Primer finder servers.	http://promoter.ics.uci.edu/Primers/

al., 1991; Makarova et al., 1992; Osborne, 1992; Li et al.,1997; Plasterer, 1997; Sze et al., 1998; Gorelenkov et al., 2001). Many programs aiding in the design of primers exist (Table 3).

GUIDELINES FOR THE DESIGN AND USE OF PRIMERS

DNA Template and oligonucleotide primers must be considered in greater detail (Linz et al., 1990). Efficacy and sensitivity of PCR largely depend on the efficiency of primers (He et al., 1994). The ability for an oligonucleotide to serve as a primer for PCR is dependent on several factors including: a) the kinetics of association and dissociation of primer-template duplexes at the annealing and extension temperatures; b) duplex stability of mismatched nucleotides and their location; and c) the efficiency with which the polymerase can recognize and extend a mismatched duplex. The primers which are unique for the target sequence to be amplified should fulfill certain criteria such as primer length, GC%, annealing and melting temperature, 5' end stability, 3' end specificity etc (Dieffenbach et al., 1995). Most of the

Table 2. PCR oligonucleotide resources.

Oligonucléotides pour la PCR	Calculation of melting point of a oligonucleotide.	http://www.citi2.fr/bio2/OligoTM.html
Oligonucleotide properties calculator	Prediction of melting temperature.	http://www.basic.nwu.edu/biotools/oligocalc.html http://www.microbiology.adelaide.edu.au/learn/oligcalc.htm
Oligonucleotide analyzer	Generates Tm, free energy, molecular weight and hairpin and dimer formation structures.	http://www.nature.com/oligonucleotide.html
Oligo Tm Determination	Prediction of Tm.	http://alces.med.umn.edu/rawtm.html
Poland	Prediction of melting temperatures of primers.	http://www.biophys.uni-duesseldorf.de/local/POLAND/poland.html
PROLIGO	Oligos parameter calculation.	http://www.gensetoligos.com/Calculation/calculation.html

Table 3. PCR primers design software for personal computer.

Software name	Descreption	www
PrimerSelect	Analyzes a template DNA sequence and chooses primer pairs for PCR and primers for DNA sequencing.	www.dnastar.com
DNASIS Max	DNASIS Max is a fully integrated program that includes a wide range of standard sequence analysis features.	http://www.medprobe.com/no/dnasis.html
Primer Premier 5	primer design for Windows and Power Macintosh.	http://www.premierbiosoft.com/primerdesign/primerdesign.html
Primer Premier:	Comprehensive primer design for Windows and Power Macintosh.	http://www.premierbiosoft.com/
NetPrimer	Comprehensive analysis of individual primers and primer pairs.	http://www.premierbiosoft.com/NetPrimer.html
Array Designer 2	For fast, effective design of specific oligos or PCR primer pairs for microarrays	http://www.premierbiosoft.com/dnamicroarray/dnamicroarray.html
Beacon Designer 2.1	Design molecular beacons and TaqMan probes for robust amplification and fluorescence in real time PCR.	http://www.premierbiosoft.com/molecular_beacons/taqman_molecular_beacons.html
GenomePRIDE 1.0	Primer design for DNA-arrays/chips.	http://pride.molgen.mpg.de/genomepride.html
Fast PCR	Software for Microsoft Windows has specific, ready-to-use templates for many PCR and sequencing applications: standard and long PCR, inverse PCR, degenerate PCR directly on amino acid sequence, multiplex PCR.	http://www.biocenter.helsinki.fi/bi/bare-1_html/manual.htm
OLIGO 6	Primer Analysis Software for Mac and Windows.	http://www.oligo.net/
Primer Designer 4	Will find optimal primers in target regions of DNA or protein molecules, amplify features in a molecule, or create products of a specified length.	http://www.scied.com/ses_pd5.htm
GPRIME	Software for primer design.	http://life.anu.edu.au/molecular/software/gprime.htm
Sarani Gold	Genome Oligo Designer is software for automatic large-scale design of optimal oligonucleotide probes for microarray experiments.	http://mail.strandgenomics.com/products/sarani/
PCR Help	Primer and template design and analysis	http://www.techne.com/CatMol/pcrhelp.htm
Genorama chip Design Software	Genorama Chip Design Software is complete set of programs required for genotyping chip design. The programs can also be bought separately.	http://www.asperbio.com/Chip_desin_soft.htm
Primer Designer	The Primer Designer features a powerful, yet extremely simple, real-time interface to allow the rapid identification of theoretical ideal primers for your PCR reactions.	http://genamics.com/expression/primer.htm
Primer Premier	Automatic design tools for PCR, sequencing or hybridization probes, degenerate primer design, Nested/Multiplex primer design, restriction enzyme analysis and more.	http://www.biotechniques.com/freesamples/itembtn21.html
PrimerDesign	DOS-program to choose primer for PCR or oligonucleotide probes.	http://www.chemie.unimarburg.de/%7Ebecker/pdhome.html

reviews on PCR optimization (Erlich et al., 1991; Dieffenbach et al., 1995; Roux, 1995) consider different parameters of PCR but generally do not discuss basic concepts of PCR primer design.

Maybe the most critical parameter for successful PCR is the design of Primers. All things being equal, a poorly designed primer can result in a PCR reaction that will not work. The primer sequence determines several things such as the length of the product, its melting temperature and ultimately the yield. A badly designed primer can result in little or no product due to non-specific amplification and/or primer-dimer formation, which can become competitive enough to suppress product formation. This application note is provided to give rules that should be taken into account when designing primers for PCR. More comprehensive coverage of this subject can be found elsewhere (Dieffenbach et al., 1995).

The sequences of the primers used for PCR amplification can have a major effect on the specificity and sensitivity of the reaction. When choosing two PCR amplification primers, the following guidelines should be considered:

Primer length: Since both specificity and the temperature and time of annealing are at least partly dependent on primer length, this parameter is critical for successful PCR (Wu et al., 1991). For broad-spectrum studies, primers of typically 18-30 nucleotides in length are the best. Primers should be at least 18 nucleotides in length to minimize the chances of encountering problems with a secondary hybridization site on the vector or insert. Primers with long runs of a single base should generally be avoided. It is especially important to avoid 4 or more G's or C's in a row.

Melting Temperature (T_m): The optimal melting temperatures for primers in the range 52-58°C, generally produce better results than primers with lower melting temperatures. Primers with melting temperatures above 65°C should also be avoided because of potential for secondary annealing. It is then advisable to do the sequencing reaction with annealing and extension at 60°C. A good working approximation of this value (generally valid for oligos in the 18–30 base range) can be calculated using the formula of Wallace et al. (1979), $T_m = 2(A+T) + 4(G+C)$. Using improved nearest-neighbor thermodynamic values given by SantaLucia et al. (1996), an estimate of melting temperature can be obtained for oligonucleotide analysis.

GC Content (Tm and Ta are Interrelated): GC% is an important characteristic of DNA and provides information about the strength of annealing. Primers should have a GC content between 45 and 60 percent (Dieffenbach et al., 1995). For primers with a G/C content of less than 50%, it may be necessary to extend the primer sequence beyond 18 bases to keep the melting temperature above the recommended lower limit of 50°C. GC content, melting temperature and annealing temperature are strictly dependent on one another (Rychlik et al., 1990).

3'-End Sequence: It is well established that the 3' terminal position in PCR primers is essential for the control of mis-priming (Kwok et al., 1990). Primers should be "stickier" on their 5' ends than on their 3' ends. A "sticky" 3' end as indicated by a high G/C content could potentially anneal at multiple sites on the template DNA. A "G" or "C" is desirable at the 3' end but the first part of this rule should apply. This GC clamp reduces spurious secondary bands (Sheffield et al., 1989).

Dimers and false priming cause misleading results: Primers should not contain complementary (palindromes) within themselves; that is, they should not form hairpins. If this state exists, a primer will fold back on itself and result in an unproductive priming event that decreases the overall signal obtained (Breslauer et al., 1986). Hairpins that form below 50°C generally are not such a problem. Primers should not contain sequences of nucleotides that would allow one primer molecule to anneal to itself or to the other primer used in PCR reactions (primer dimer formation).

Specificity: As mentioned above, primer specificity is at least partly dependent on primer length. It is evident that there are many more unique 24 base oligos than there are 15 base pair oligos. However, primers must be chosen so that they have a unique sequence within the template DNA that is to be amplified. A primer designed with a highly repetitive sequence will result in a smear when amplifying genomic DNA. However, the same primer may give a single band if a single clone from a genomic library is amplified.

Degenerate Primers: Degeneracy in primer sequence should also be taken into consideration. Degenerate primers based on the amino acid sequence of conserved regions were also used to search for members of a gene family (Wilks et al., 1989). Computer programs have also been developed specifically for degenerate primer design (Chen and Zhu, 1997).

Complementary primer sequences: Primers need to be designed with absolutely no intra-primer homology beyond 3 base pairs. If a primer has such a region of self-homology, "snap back" can occur. Another related danger is inter-primer homology: partial homology in the middle regions of two primers can interfere with hybridization. If the homology should occur at the 3' end of either primer, primer dimer formation will occur.

Other recommendations: The concentration of primer in amplification reaction should be between 0.1 and 0.5 µm. If possible, a computer search should be conducted against the vector and insert DNA sequences to verify that the primer and especially the 8-10 bases of its 3' end

are unique. Inosine should not be included in sequencing primers. They either do not work or give poor cycle sequencing results. The design of PCR and DNA sequencing primers follows very similar guidelines. Even though primer characteristics can be visually inspected for the presence of the elements listed above, a number of computer programs that have been developed use several of these guidelines for primer selection.

CONCLUSION

The key to the PCR lies in the design of the two oligonucleotide primers. It is essential that care is taken in the design of primers for PCR. Several parameters including the length of the primer, %GC content and the 3' sequence need to be optimized for successful PCR. Certain of these parameters can be easily by hand optimized while others are best done with marketable computer programs. The increasing use of information from the internet and the sequences held in gene databases are practical starting points when designing primers and reaction conditions for the PCR. A number or software packages such as Oligo, Primer etc. have allowed the process of primer design to be less troublesome. It is also possible to include more than one set of primers in a PCR.

REFERENCES

Binas M (2000). Designing PCR primers on the web. Biotechniques 29: 988-990.

Breslauer KJ, Ronald F, Blocker H, Marky LA (1986). Predicting DNA duplex stability from the base sequence. Proc. Natl. Acad. Sci. 83: 3746-3750.

Chen H, Zhu G (1997). Computer program for calculating the melting temperature of degenerate oligonucleotides used in PCR or hybridization. Biotechniques 22: 1158-1160

Dieffenbach CW, Lowe TMJ, Dveksler GS (1995). General Concepts for PCR Primer Design. In: PCR Primer, A Laboratory Manual, Dieffenbach CW, Dveksler GS Ed., Cold Spring Harbor Laboratory Press, New York, 133-155.

Erlich HA, Gelfand D, Sninsky JJ (1991). Recent advances in the polymerase chain reaction. Science 252: 1643-1651.

Gorelenkov V, Antipov A, Lejnine S, Darasella N, Yuryev A (2001). Set of novel tools for PCR primer design. Biotechniques 31:1326-1330.

He Q, Marjamaki M, Soini H, Mertsola J, Viljanen MK (1994). Primers are decisive for sensitivity of PCR. Biotechniques. 17: 82-87.

Kwok S, Kellogg DE, McKinney N, Spasic D, Goda L, Levenson C, Sninsky J (1990). Effects of primer-template mismatches on the polymerase chain reaction: Human Immunodeficiency Virus 1 model studies. Nucleic Acids Res. 18: 999-1005.

Li PKC, Kupfer CJ, Davies D, Burbee G, Evans A, Garner HR (1997). PRIMO: A primer design program that applies base quality statistics for automated large-scale DNA sequencing. Genomics 40:476-485.

Linz U, Delling U, Rubsamem-Waigmann (1990). Systematic studies on parameters influencing the performance of the polymerase chain reaction. J. Clin. Chem. Clin. Biochem. 28: 5-13.

Lowe T, Sharefkin J, Yang SQ, Dieffenbach CW (1990). A computer program for selection of oligonucleotide primers for polymerase chain reaction. Nucleic Acids Res. 18: 1757-1761.

Lucas K, Busch M, Mossinger S, Thompson JA (1991). An improved microcomputer program for finding gene or gene family-specific oligonucleotides suitable as primers for polymerase chain reactions or as probes. Comput. Appl. Biosci. 7: 525-529.

Makarova KS, Mazin AV, Wolf YI, Soloviev VV (1992). DIROM: An experimental design interactive system for directed mutagenesis and nucleic acid engineering. Comput. Appl. Biosci. 8: 425-431.

O'Hara PJ, Venezia D (1991). PRIMEGEN: a tool for designing primers from multiple alignments. CABIOS 7: 533-534.

Osborne BI (1992). Hyper PCR: A Macintosh Hypercard program for determination of optimal PCR annealing temperature. Comput. Appl. Biosci. 8: 33-34.

Plasterer TN (1997). PRIMERSELECT: Primer and probe design. Methods Mol. Biol. 70: 291-302.

Roux KH (1995). Optimization and troubleshooting in PCR in: PCR methods and applications. Cold spring Harbor laboratory. 5185-5194.

Rychlik W, Rhoads RE (1989). A computer program for choosing optimal oligonucleotides for filter hybridization, sequencing and *in vitro* amplification of DNA. Nucleic Acids Res. 17: 8543-8551.

Rychlik W, Spencer WJ, Rhoads RE (1990). Optimisation of annealing temperature for DNA amplification in vitro. Nucleic Acids Res. 18: 6409-6412.

SantaLucia JJr, Allawi HAT, Seneviratne PA (1996). Improved Nearest-Neighbor parameters for predicting DNA duplex stability. Biochemistry 35: 3555-3562.

Sheffield VC, Cox DR, Lerman LS, Myers RM (1989). Attachment of a 40 base pair G+C rich sequence (GC-clamp) to genomic DNA fragments by polymerase chain reaction results in improved detection of single base changes. Proc. Natl. Acad. Sci. 86: 232-236.

Singh VK, Kumar A (2001). PCR Primer Design. Mol. Biol. Today 2: 27-32.

Sze SH, Raytberg MA, Gelfand MS, Mironov AA, Astakhova TV, Pevzner PA (1998). Algorithms and software for support of gene identification experiments. Bioinformatics 14: 14-19.

Tamura T, Holbrook SR, Kim SH (1991). A Macintosh computer program for designing DNA sequences that for specific peptides and proteins. Biotechniques 10: 782-784.

Wallace RB, Shaffer J, Murphy RF, Bonner J, Hirose T, Itakura K (1979). Hybridization of synthetic oligodeoxyribonucleotides to phi chi 174 DNA: the effect of single base pair mismatch. Nucleic Acids Res. 6: 3543-3557.

Wilks AF, Kurban RR, Hovens CM, Ralph SJ (1989). The application of the polymerase chain reaction to cloning members of the protein tyrosine kinase family. Gene 85: 67-74.

Wu DYW, Ugozzoli L, Pal BK, Qian J, Wallace RB (1991). The effect of temperature and oligonucleotide primer length on specificity and efficiency of amplification by the polymerase chain reaction. DNA Cell Biol. 10: 233-238.

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Comparison of Three PCR Primer Sets for Identification of *vanB* Gene Carriage in Feces and Correlation with Carriage of Vancomycin-Resistant Enterococci: Interference by *vanB*-Containing Anaerobic Bacilli

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We assessed the sensitivities and specificities of three previously described PCR primers on enrichment broth cultures of feces for the accurate detection of fecal carriage of vancomycin-resistant enterococci (VRE). In addition, we investigated specimens that were *vanB* PCR positive but VRE culture negative for the presence of other *vanB*-containing pathogens. Feces from 59 patients (12 patients carrying *vanB Enterococcus faecium* strains and 47 patients negative for VRE carriage) were cultured for 36 h in aerobic brain heart infusion (BHI) broth, anaerobic BHI (AnO_2BHI) broth, or aerobic Enterococcosel (EC) broth. DNA was extracted from the cultures and tested for the presence of *vanB* by using the PCR primers of Dutka-Malen et al. (S. Dutka-Malen, S. Evers, and P. Courvalin, J. Clin. Microbiol. 33:24–27, 1995), Bell et al. (J. M. Bell, J. C. Paton, and J. Turnidge, J. Clin. Microbiol. 36:2187–2190, 1998), and Stinear et al. (T. P. Stinear, D. C. Olden, P. D. R. Johnson, J. K. Davies, and M. L. Grayson, Lancet 357:855–856, 2001). The sensitivity (specificity) of PCR compared with the results of culture on BHI, AnO_2BHI, and EC broths were 67% (96%), 50% (94%), and 17% (100%), respectively, with the primers of Dutka-Malen et al.; 92% (60%), 92% (45%), and 92% (83%), respectively, with the primers of Bell et al.; and 92% (49%), 92% (43%), and 100% (51%) respectively, with the primers of Stinear et al. The primers of both Bell et al. and Stinear et al. were significantly more sensitive than those of Dutka-Malen et al. in EC broth ($P = 0.001$ and $P < 0.001$, respectively). The poor specificities for all primer pairs were due in part to the isolation and identification of six anaerobic gram-positive bacilli, *Clostridium hathewayi* ($n = 3$), a *Clostridium innocuum*-like organism ($n = 1$), *Clostridium bolteae* ($n = 1$), and *Ruminococcus lactaris*-like ($n = 1$), from five fecal specimens that were *vanB* positive but VRE culture negative. All six organisms were demonstrated to contain a *vanB* gene identical to that of VRE. *VanB*-containing bowel anaerobes may result in false-positive interpretation of PCR-positive fecal enrichment cultures as VRE, regardless of the primers and protocols used.

Minimization of the risk of colonization and infection by vancomycin-resistant enterococci (VRE) is a major infection control goal in Australia, as elsewhere. However, the prolonged time that it takes to obtain results by routine microbiological screening techniques often results in delays in implementation of appropriate infection control measures, such as separating patients into cohorts (12, 21). Six different vancomycin resistance gene (*van*) operons (*vanA*, *vanB*, *vanC*, *vanD*, *vanE*, and *vanG*) are known to mediate glycopeptide resistance, with three alleles described for the *vanB* ligase gene (*vanB1*, *vanB2*, and *vanB3*) (6, 16, 23). The most commonly recognized pathogenic genotype in Australia is *vanB*, the *vanB2* allele of which predominates (A. Berry, H. Sundqvist, J. Bell, and J. Turnidge, Abstr. 3rd Annu. Sci. Meet. Aust. Soc. Antimicrob., abstr. 22, Feb. 2002).

Present PCR protocols for the direct detection of VRE in clinical specimens or enrichment broth cultures of these specimens rely on the detection of *van* genes, specifically, *vanA* or *vanB* (18, 22, 25, 28, 29). These methods are not dependent on the identity of the host organism, and as such, the identification of VRE can only be inferred, as the specimen contains a mixed bacterial population. Since the *vanB* gene has been described in *Streptococcus* species and more recently in *Eggerthella lenta* and three *Clostridium* species isolated from human feces, the presence of *vanB* in feces does not necessarily imply the presence of VRE (19, 27, 31). For this reason, we assessed the sensitivities and specificities of three frequently used primers under different growth and PCR conditions for the accurate identification of VRE in fecal specimens from patients for whom knowledge about their VRE carriage status was known.

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MATERIALS AND METHODS

Clinical specimens and bacterial strains. Fecal samples were collected from 59 patients who were previously well characterized with regard to the presence or absence of fecal VRE carriage. Fecal specimens were collected from 53 hemodialysis patients; of these, 6 patients were known to be fecally colonized

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with *vanB E. faecium*, while 47 patients had repeatedly been shown to be VRE negative. Feces were also collected from six nonhemodialysis patients at our hospital who were known to be fecally colonized with VRE (all with *vanB E. faecium*) as a result of tests with at least two recent consecutive fecal specimens. Thus, all 12 VRE-colonized patients carried *vanB E. faecium*. All fecal specimens were collected and then stored at −80°C. Control enterococcal strains used for PCR were an *Enterococcus faecalis* strain containing *vanB* (ATCC 51299), a vancomycin-sensitive *E. faecalis* strain (ATCC 29212), and clinical isolates of *vanA E. faecium* and *vanB E. faecium*, each of which was obtained at our institution.

Enrichment broth cultures of feces and isolation of VRE. All fecal specimens were assessed for the presence of VRE both by direct culture on agar and by culture in enrichment broth. Enrichment broth cultures of thawed fecal specimens were obtained by the following protocol. A sterile 50-μl plastic loopful of fecal material was used to inoculate each of the following broths (6 ml each): brain heart infusion (BHI) broth and Enterococcosel (EC) broth (both of which were incubated aerobically) and BHI broth with 0.5% yeast extract, 0.1% sodium thioglycolate, 0.25% glucose, and 0.5 μg of hemin ml^{-1} (AnO_2BHI broth; which was incubated anaerobically). All broth cultures were incubated at 35°C for 36 h. Subsequently, all VRE culture-positive specimens were recultured by the same method, but with assessment at both 17 and 36 h.

Isolation of VRE was performed as follows. All fecal specimens and enrichment broth cultures were plated on EC agar containing vancomycin at 6 μg/ml and were incubated at 35°C for 48 to 72 h. Esculin-positive colonies were investigated as described previously (21). The identities of isolates provisionally identified as *Enterococcus faecium* or *E. faecalis* were confirmed by multiplex PCR for the D-Ala–D-Ala ligase gene (7). The glycopeptide resistance genotype was determined by multiplex PCR for *vanA*, *vanB*, and *vanC* (2).

Detection of *van* genes in enrichment broth cultures of feces. DNA was extracted from a 500-μl volume of fecal enrichment broth as described by Stinear et al. (30). DNA samples were frozen at −20°C until they were required. A 5-μl volume of DNA was tested by PCR for *vanA* and *vanB* in a 50-μl reaction mixture containing 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 2 mM $MgCl_2$; 200 μM each dATP, dTTP, dGTP, and dCTP; 0.25 μM each primer; and 0.25 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, by Roche Molecular Systems, Inc., Branchburg, N.J.). PCR cycling was performed on a GeneAmp2700 thermal cycler (Applied Biosystems). Known positive and negative controls (including multiple controls consisting of sterile water [i.e., no DNA]) were included in each PCR run. The PCR products were resolved by electrophoresis on agarose-Tris-acetate-EDTA gels containing 0.5 μg of ethidium bromide per ml. The gels were visualized and photographed with a Fluor-S MultiImager and Quantity One quantitation software (version 4; Bio-Rad Laboratories, Hercules, Calif.).

The following three different primer sets were assessed. The PCR protocol of Stinear et al. (31) was performed with primers VanB-P1 and VanB-P2, designed for the detection of *vanB*; the primers and PCR protocol are referred to as "Stinear." The cycling conditions for the Stinear protocol were 95°C for 10 min, followed by 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min for 5 cycles; 94°C for 20 s, 58°C for 30 s, and 72°C for 40 s for 35 cycles; and 72°C for 5 min (T. Stinear, personal communication). The PCR protocol of Bell et al. (2) was performed with primers VanABF, VanAR, and VanBR, designed for the detection of *vanA* and *vanB* by multiplex PCR; the primers and PCR protocol are referred to as "Bell." The cycling conditions were 95°C for 10 min, followed by 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min for 40 cycles and 72°C for 5 min (J. Bell, personal communication). The PCR protocol of Dutka-Malen et al. (7) was performed with primers, A_1, A_2, B_1, and B_2, designed for the detection of *vanA* and *vanB*; the primers and PCR protocol are referred to as "Dutka-Malen." The exception to the Dutka-Malen protocol was that 0.5 μM each forward and reverse primers was used. The cycling conditions were as follows: 95°C for 10 min, followed by 94°C for 1 min, 54°C for 1 min, and 72°C for 1 min for 40 cycles and 72°C for 5 min (7). This protocol was repeated by using an annealing temperature of 50°C because of the poor sensitivity obtained by the original method.

Isolation of anaerobes. To optimize the isolation of vancomycin-resistant anaerobes from *vanB*-positive, VRE culture-negative feces, a swab of fecal material was subcultured in 6-ml volumes of AnO_2BHI broth containing vancomycin at 10 μg/ml and incubated anaerobically at 35°C for 48 h, as described above. These cultures were then serially diluted in AnO_2BHI broth, and 100-μl aliquots were plated onto horse blood agar containing vancomycin at 6 μg/ml (HBA-V) and colistin-nalidixic acid blood agar to obtain single colonies. The plates were incubated anaerobically at 35°C for 48 h. Single colonies from both media were assessed for purity on HBA-V. Isolates were investigated by Gram staining, and their identities were determined by sequencing of their 16S rRNA genes. The presence of *vanB* was tested by PCR with the Bell primers, and the gene was sequenced for confirmation (see below).

DNA sequencing and analysis. DNA for sequencing was prepared from pure isolates of anaerobes by using the DNeasy Tissue kit (Qiagen Pty. Ltd., Clifton Hill, Victoria, Australia) and the modified protocol for isolation of genomic DNA from gram-positive bacteria, as described in the instructions of the manufacturer. A 1,376-bp region of the 16S rRNA gene was generated with the primers described by Gurtler et al. (13), and a 2,917-bp region of the *vanB* operon encompassing the *vanB* ligase gene was generated with primers 5′-TGC TTCCAATGAGACGGGCG-3′ and 5′-CTTTGTGCCGATGATGCGAT-3′ by PCR with the Expand Long Template PCR system (Roche Applied Science, Mannheim, Germany). PCR elongation times and temperatures were adjusted according to the expected size of the product and the nucleotide sequences of the primers, respectively, as recommended by the manufacturer. The PCR products to be sequenced were purified with a QIAquick PCR purification kit (Qiagen Pty. Ltd.). The sequences of the PCR products were determined by cycle sequencing with the same primers used for the PCR and the ABI Prism BigDye Terminator (version 3.0) Ready Reaction Cycle Sequencing kit (Applied Biosystems) with an ABI Prism 3100 genetic analyzer. The chromatograms were read and the contiguous sequences were constructed with Vector NTI Advance software (version 8.0; Informax Inc., Bethesda, Md.). The sequences were compared for homology with sequences in the GenBank, EMBL, DDBJ, and PDB databases by using the BLASTN local alignment search tool (1) and the National Center for Biotechnology Information website (www. ncbi.nlm.nih.gov). The sequences of the *vanB* ligase gene were aligned by using the AlignX module of Vector NTI Advance software (version 8.0) and the CLUSTAL W algorithm (33).

Statistical analysis. Statistical comparisons of the sensitivity and the specificity of each primer for the identification of the presence of VRE were undertaken by chi-square analysis.

Nucleotide sequence accession numbers. The nucleotide sequences of the 16S rRNA genes from isolates MLG856-1, MLG856-2, MLG480, MLG080-1, MLG080-3, MLG392, and MLG661 have been submitted to GenBank and given consecutive accession numbers AY653230 to AY653236, respectively. The nucleotide sequences of the *vanB* ligase genes from these isolates have also been submitted to GenBank and given the consecutive accession numbers AY655710 to AY655716, respectively.

RESULTS

A total of 59 fecal specimens, 12 of which were VRE culture positive and 47 of which were VRE culture negative, were examined for the presence of *vanB* by PCR following enrichment broth culture. The sensitivity and specificity of each of the three PCR primer sets for the accurate detection of the presence of fecal VRE carriage with each of the three enrichment broth cultures are shown in Table 1. The Stinear and Bell primers were more sensitive than the Dutka-Malen primers for the detection of VRE under all growth conditions assessed, although this difference was statistically significant only in EC broth ($P < 0.001$ and $P = 0.001$, respectively; Table 1). The optimal balance between sensitivity and specificity appeared to be best achieved with the Bell primers for the fecal cultures in EC broth. Notably, the sensitivity of the Bell primer and PCR protocol did not change when EC broth cultures incubated for only 17 h were used (data not shown). In contrast, the Dutka-Malen primer set appeared to be less sensitive, even when the annealing temperature used in the PCR was decreased from 54 to 50°C.

Both the Bell and Stinear primer sets detected *vanB* genes in a large number of fecal specimens that were culture negative for VRE. This was especially notable when AnO_2BHI enrichment broth was used, with which 26 and 27 of the 47 VRE culture-negative specimens had detectable *vanB* genes when these two primer sets were used, respectively. Attempts were made to isolate the source of the *vanB* signal in these 27 specimens. For 18 specimens the *vanB* gene signal was lost

TABLE 1. Sensitivity and specificity of *vanB* PCR protocols for detection of VRE in feces cultured in enrichment broth for 36 h

Primer and PCR protocol	BHI broth cultures				AnO_2BHI broth cultures				EC broth cultures			
	No. of PCR-positive specimens[a]		Sensitivity (%)	Specificity (%)	No. of PCR-positive specimens		Sensitivity (%)	Specificity (%)	No. of PCR-positive specimens		Sensitivity (%)	Specificity (%)
	VRE + (n = 12)	VRE − (n = 47)			VRE + (n = 12)	VRE − (n = 47)			VRE + (n = 12)	VRE − (n = 47)		
Stinear	11	24	92[b,c]	49	11	27	92[b]	43	12	23	100[d,e]	51[f]
Bell	11	19	92	60	11	26	92[b]	45	11	8	92[g,h]	83[i,j]
Dutka-Malen	8	2	67[c]	96[k]	6	3	50[b]	94[k]	2	0	17[d,g]	100[i,j]
Dutka-Malen[l]	6	0	50[b]	100[k]	6	2	50[b]	96[k]	4	1	33[e,h]	98[i,j]

[a] Number of specimens giving the expected PCR product. VRE +, VRE positive; VRE −, VRE negative.
[b] Difference not statistically significant (P = 0.07).
[c] Difference not statistically significant (P = 0.32).
[d] Stinear primers significantly more sensitive than Dutka-Malen primers (P < 0.001).
[e] Stinear primers significantly more sensitive than Dutka-Malen primers (P = 0.002).
[f] Dutka-Malen primers significantly more specific than Stinear primers (P < 0.001).
[g] Bell primers significantly more sensitive than Dutka-Malen primers (P = 0.001).
[h] Bell primers significantly more sensitive than Dutka-Malen primers (P = 0.01).
[i] Dutka-Malen primers significantly more specific than Bell primers (P = 0.01).
[j] Dutka-Malen primers significantly more specific than Bell primers (P = 0.035).
[k] Dutka-Malen primers significantly more specific than either Stinear or Bell primers (P < 0.001).
[l] Dutka-Malen primer and PCR protocol repeated with an annealing temperature of 50°C.

when the positive broth was further subcultured in AnO_2BHI broth under anaerobic conditions. For four specimens, the *vanB* signal persisted after this subculture, but no organisms containing *vanB* could be isolated. A total of seven different *vanB*-containing organisms were isolated from the remaining five *vanB* PCR-positive, VRE culture-negative specimens (Table 2). For one specimen, an *E. faecium* isolate not previously isolated by routine culture methods and an isolate closely related to *Clostridium hathewayi* (98% 16S rRNA gene sequence identity) were identified. Of the remaining five organisms isolated, two were related to *C. hathewayi* (98% 16S rRNA gene identity), one was related to *Clostridium bolteae* (98% 16S rRNA gene identity), and one was most likely a new species with only 91% 16S rRNA gene identity to *Clostridium innocuum*. The remaining isolate was a gram-variable coccobacillus with 91% identity by 16S rRNA gene analysis to the *Ruminococcus lactaris* 16S rRNA gene sequence (Table 2). The entire *vanB* ligase genes from all seven isolates were sequenced. For six of these isolates the *vanB* ligase gene sequence was 99% identical to the *vanB2* sequences of *E. faecium* C68 (3) and *E. faecalis* 268-10 (11), while for MLG480 the *vanB* ligase gene sequence was 99% identical to the *vanB1* sequence of *E. faecalis* V583 (9) and only 97% identical to the *vanB2* sequence.

DISCUSSION

The rapid detection of VRE colonization may have important infection control implications in centers where VRE-colonized patients are separated into cohorts to minimize nosocomial transmission (12, 21). Although conventional culture methods for the detection of VRE may be sensitive, they frequently take 3 to 5 days to confirm the presence or absence of VRE (5, 8, 14, 18, 25, 28). A number of investigators have attempted the direct detection of *van* genes in either feces or enrichment broth cultures of feces as a surrogate means of identifying VRE carriage, with variable results (18, 22, 24, 25, 28). The sensitivities and specificities of such methods depend on the type of *van* genotype, the PCR primers used, and the nature of the enrichment broth.

In our study we found that for the detection of *vanB* VRE, PCR with the Bell primers with DNA extracted from 36-h EC broth cultures of feces provided the best balance between sensitivity (92%) and specificity (83%) compared with the results of culture. Lu et al. (18), who designed novel primers for the detection of *vanA* and *vanB* and who developed a multiplex PCR for the detection of *van* genes directly from EC enrichment broths containing 8 μg of vancomycin per ml, reported a 97.9% sensitivity for VRE detection. Similarly, Palladino et al.

TABLE 2. Characteristics of vancomycin-resistant organisms isolated from five fecal specimens that contained *vanB* genes after anaerobic subculture, but no easily identifiable VRE, by routine methods

Specimen	Isolate	Gram stain result	Identification based on 16S rRNA gene (accession no.)	% Sequence identity
1	MLG856-1	Positive, bacillus	*Clostridium hathewayi* (AJ311620)	98
	MLG856-2	Positive, coccus	*Enterococcus faecium* (AF070223)	99
2	MLG480	Negative,[a] bacillus	*Clostridium innocuum* (AF028352)	91
3	MLG080-1	Variable,[a] bacillus[a]	*Clostridium bolteae* (AJ08452)	98
	MLG080-3	Variable, coccobacillus[b]	*Ruminococcus lactaris* (L76602)	91
4	MLG392	Positive, bacillus	*Clostridium hathewayi* (AJ311620)	98
5	MLG661	Positive, bacillus	*Clostridium hathewayi* (AJ311620)	98

[a] Not all species classified in the genus *Clostridium* stain gram positive, and staining may vary depending on the medium used and the age of the culture (15).
[b] Anaerobic gram-positive cocci may be elongated and resemble coccobacilli and lose their positivity with age (20).

(22) reported a sensitivity of detection of 88% with the primers designed by Dutka-Malen et al. (7) for a *vanA-vanB* multiplex real-time LightCycler PCR with EC enrichment broths containing 8 μg of vancomycin per ml. Notably, however, neither of those studies considered the sensitivity of detection of each genotype separately. In comparison, we found the Dutka-Malen primers to be insensitive (regardless of whether we used an annealing temperature of 54 or 50°C), detecting only 17 to 67% of *vanB* VRE culture-positive specimens, depending on the enrichment broth used.

Interestingly, we found that the two most sensitive methods (those with the Bell and Stinear primers) resulted in a substantial number of false-positive results in which no VRE could be identified. Paule et al. (24) reported similar results when they compared culture with a direct multiplex PCR with the Dutka-Malen *vanA*- and *vanB*-specific primers for the detection of VRE from rectal swabs. Similarly, Sloan et al. (29) reported a higher frequency of discordant results for *vanB* gene detection when they compared a LightCycler *vanA-vanB* detection assay with culture for the direct detection of VRE from perianal stool swab specimens. Such results may be due to the presence of nonviable VRE, viable nonculturable VRE, or a low burden of VRE (17, 24, 29). However, the presence of nonenterococcal reservoirs of *vanB* has been described previously (19, 27, 31). Notably, we isolated five anaerobic gram-positive bacilli related to the clostridia and one *Ruminococcus* sp. from five PCR-positive, VRE culture-negative fecal specimens. These six anaerobes contained a *vanB* ligase gene identical to that identified in *vanB*-containing VRE strains. One of these isolates was obtained from a fecal specimen that also contained low numbers of *vanB E. faecium*. Since approximately 40% of the bacterial community of the bowel has yet to be cultivated in vitro, presumably due to their fastidious growth and nutritional requirements, the identification of other vancomycin-resistant anaerobes remains a possibility (32).

It is important that the majority of our fecal specimens were obtained from patients requiring long-term hemodialysis, so we are uncertain whether factors related to their underlying illness and/or treatment may have influenced the likelihood that these patients were fecally colonized with *vanB*-containing anaerobes. Moreover, as all our patients with VRE were colonized with *vanB*-type VRE, we cannot extrapolate our findings regarding the sensitivity and specificity of each of the PCR primers assessed to other *van* genotypes, particularly *vanA*. However, given the reports of nonenterococcal reservoirs of *vanA*, it will be important to assess the sensitivity and specificity of PCR for the detection of *vanA* in a mixed culture for the identification of *vanA*-type VRE (4, 10, 26). Regardless of these caveats, clinicians and infection control practitioners who rely on molecular analysis of feces to rapidly identify patients who are fecally colonized with VRE need to be aware of the potential role of *vanB* gene-containing anaerobes in causing false-positive interpretations of the presence of VRE.

ACKNOWLEDGMENTS

We thank S. Xie and K. K. Pertile for generous technical assistance.

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REFERENCES

1. **Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25:3389–3402.
2. **Bell, J. M., J. C. Paton, and J. Turnidge.** 1998. Emergence of vancomycin-resistant enterococci in Australia: phenotypic and genotypic characteristics of isolates. J. Clin. Microbiol. 36:2187–2190.
3. **Carias, L. L., S. D. Rudin, C. J. Donskey, and L. B. Rice.** 1998. Genetic linkage and cotransfer of a novel, vanB-containing transposon (Tn*5382*) and a low-affinity penicillin-binding protein 5 gene in a clinical vancomycin-resistant *Enterococcus faecium* isolate. J. Bacteriol. 180:4426–4434.
4. **Centers for Disease Control and Prevention.** 2002. *Staphylococcus aureus* resistant to vancomycin—United States, 2002. Morb. Mortal. Wkly. Rep. 51:565–567.
5. **D'Agata, E. M. C., S. Gautam, W. K. Green, and Y.-W. Tang.** 2002. High rate of false-negative results of the rectal swab culture method in detection of gastrointestinal colonization with vancomycin-resistant enterococci. Clin. Infect. Dis. 34:167–172.
6. **Dahl, K. H., G. S. Simonsen, O. Olsvik, and A. Sundsfjord.** 1999. Heterogeneity in the *vanB* gene cluster of genomically diverse clinical strains of vancomycin-resistant enterococci. Antimicrob. Agents Chemother. 43:1105–1110.
7. **Dutka-Malen, S., S. Evers, and P. Courvalin.** 1995. Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. J. Clin. Microbiol. 33:24–27. (Erratum, 33: 1434.
8. **Edberg, S. C., C. J. Hardalo, C. Kontnick, and S. Campbell.** 1994. Rapid detection of vancomycin-resistant enterococci. J. Clin. Microbiol. 32:2182–2184.
9. **Evers, S., P. E. Reynolds, and P. Courvalin.** 1994. Sequence of the *vanB* and *ddl* genes encoding D-alanine: D-lactate and D-alanine: D-alanine ligases in vancomycin resistant *Enterococcus faecalis* V583. Gene 140:97–102.
10. **Fontana, R., M. Ligozzi, C. Pedrotti, E. M. Padovani, and G. Cornaglia.** 1997. Vancomycin-resistant *Bacillus circulans* carrying the *vanA* gene responsible for vancomycin-resistance in enterococci. Eur. J. Clin. Microbiol. Infect. Dis. 16:473–474.
11. **Garnier, F., S. Taourit, P. Glaser, P. Courvalin, and M. Galimand.** 2000. Characterization of transposon Tn*1549*, conferring VanB-type resistance in *Enterococcus* spp. Microbiology 146:1481–1489.
12. **Grayson, M. L., E. A. Grabsch, P. D. R. Johnson, D. Olden, M. Aberline, H. Y. Li, G. Hogg, M. Abbott, and P. G. Kerr.** 1999. Outcome of a screening program for vancomycin-resistant enterococci in a hospital in Victoria. Med. J. Aust. 171:133–136.
13. **Gurtler, V., V. A. Wilson, and B. C. Mayall.** 1991. Classification of medically important clostridia using restriction endonuclease site differences of PCR-amplified 16S rDNA. J. Gen. Microbiol. 137:2673–2679.
14. **Jayaratne, P., and C. Rutherford.** 1999. Detection of clinically relevant genotypes of vancomycin-resistant enterococci in nosocomial surveillance specimens by PCR. J. Clin. Microbiol. 37:2090–2092.
15. **Johnson, M. J., E. Thatcher, and M. E. Cox.** 1995. Techniques for controlling variability in Gram staining of obligate anaerobes. J. Clin. Microbiol. 33: 755–758.
16. **Kak, V., and J. W. Chow.** 2002. Acquired antibiotic resistances in enterococci, p. 355–383. *In* D. B. Clewell, P. Courvalin, G. M. Dunny, B. E. Murray, and L. B. Rice (ed.), The enterococci. American Society for Microbiology, Washington, D.C.
17. **Lleò, M. M., B. Bonato, C. Signoretto, and P. Canepari.** 2003. Vancomycin resistance is maintained in enterococci in the viable but nonculturable state and after division is resumed. Antimicrob. Agents Chemother. 47:1154–1156.
18. **Lu, J. J., C. L. Perng, T. S. Chiueh, S. Y. Lee, C. H. Chen, F. Y. Chang, C. C. Wang, and W. M. Chi.** 2001. Detection and typing of vancomycin-resistance genes of enterococci from clinical and nosocomial surveillance specimens by multiplex PCR. Epidemiol. Infect. 126:357–363.
19. **Mevius, D., L. Devriese, P. Butaye, P. Vandamme, M. Verschure, and K. Veldman.** 1998. Isolation of glycopeptide resistant *Streptococcus gallolyticus* strains with *vanA*, *vanB*, and both *vanA* and *vanB* genotypes from faecal samples of veal calves in The Netherlands. J. Antimicrob. Chemother. 42: 275–276.
20. **Moncla, B. J., and S. L. Hillier.** 2003. *Peptostreptococcus*, *Propionibacterium*, *Lactobacillus*, *Actinomyces*, and other non-spore-forming anaerobic gram-positive bacteria, p. 857–879. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.), Manual of clinical microbiology, 8th ed. American Society for Microbiology, Washington, D.C.
21. **Padiglione, A., R. Wolfe, E. Grabsch, D. Olden, S. Pearson, C. Franklin, D. Spelman, B. Mayall, P. D. R. Johnson, and M. L. Grayson.** 2003. Risk factors for new detection of vancomycin-resistant enterococci in acute-care hospitals that employ strict infection control procedures. Antimicrob. Agents Chemother. 47:2492–2498.
22. **Palladino, S., I. D. Kay, J. P. Flexman, I. Boehm, A. M. G. Costa, E. J. Lambert, and K. J. Christiansen.** 2003. Rapid detection of *vanA* and *vanB*

genes directly from clinical specimens and enrichment broths by real-time multiplex PCR assay. J. Clin. Microbiol. 41:2483–2486.
23. Patel, R., J. R. Uhl, P. Kohner, M. K. Hopkins, J. M. Steckelberg, B. Kline, and F. R. Cockerill III. 1998. DNA sequence variation within *vanA*, *vanB*, *vanC-1*, and *vanC-2/3* genes of clinical *Enterococcus* isolates. Antimicrob. Agents Chemother. 42:202–205.
24. Paule, S., W. E. Trick, F. C. Tenover, M. Lankford, S. Cunningham, V. Stosor, R. L. Cordell, and L. R. Peterson. 2003. Comparison of PCR assay to culture for surveillance detection of vancomycin resistant enterococci. J. Clin. Microbiol. 41:4805–4807.
25. Petrich, A. K., K. E. Luinstra, D. Groves, M. A. Chernesky, and J. B. Mahony. 1999. Direct detection of *vanA* and *vanB* genes in clinical specimens for rapid identification of vancomycin resistant enterococci (VRE) using multiplex PCR. Mol. Cell. Probes 13:275–281.
26. Power, E. G. M., Y. H. Abdulla, H. G. Talsania, W. Spice, S. Asthithan, and G. L. French. 1995. *van A* genes in vancomycin-resistant clinical isolates of *Oerskovia turbata* and *Arcanobacterium* (*Corynebacterium*) *haemolyticum*. J. Antimicrob. Chemother. 36:595–606.
27. Poyart, C., C. Pierre, G. Quesne, B. Pron, P. Berche, and P. Trieu-Cuot. 1997. Emergence of vancomycin resistance in the genus *Streptococcus*: characterization of a *vanB* transferable determinant in *Streptococcus bovis*. Antimicrob. Agents Chemother. 41:24–29.
28. Satake, S., N. Clark, D. Rimland, F. S. Nolte, and F. C. Tenover. 1997. Detection of vancomycin-resistant enterococci in fecal samples by PCR. J. Clin. Microbiol. 35:2325–2330.
29. Sloan, L. M., J. R. Uhl, E. A. Vetter, C. D. Schleck, W. S. Harmsen, J. Manahan, R. L. Thompson, J. E. Rosenblatt, and F. R. Cockerill III. 2004. Comparison of the Roche LightCycler *vanA/vanB* detection assay and culture for detection of vancomycin-resistant enterococci from perianal swabs. J. Clin. Microbiol. 42:2636–2643.
30. Stinear, T., J. K. Davies, G. A. Jenkin, F. Portaels, B. C. Ross, F. Oppedisano, M. Purcell, J. A. Hayman, and P. D. R. Johnson. 2000. A simple PCR method for rapid genotype analysis of *Mycobacterium ulcerans*. J. Clin. Microbiol. 38:1482–1487.
31. Stinear, T. P., D. C. Olden, P. D. R. Johnson, J. K. Davies, and M. L. Grayson. 2001. Enterococcal *vanB* resistance locus in anaerobic bacteria in human faeces. Lancet 357:855–856.
32. Tannock, G. W., K. Munro, H. J. M. Harmsen, G. W. Welling, J. Smart, and P. K. Gopal. 2000. Analysis of the fecal microflora of human subjects consuming a probiotic product containing *Lactobacillus rhamnosus* DR20. Appl. Environ. Microbiol. 66:2578–2588.
33. Thompson, J. D., D. G. Higgins, and T. J. Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multi-sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22:4673–4680.

they are earned. Nintendo complains that Mr. Willis' analysis is the equivalent of assuming royalty payments were made on a daily basis, and that his method artificially inflates prejudgment interest by $15,000,000. Alpex responds that its calculation conservatively estimates a quarterly royalty payment schedule, and that such a schedule is reasonable in light of the fact that Nintendo's own license agreements generally specified such payments. To make its point, Alpex compares the prejudgment interest resulting from its mid-year calculation with higher figures allegedly resulting from a quarterly payment assumption. Unfortunately, as Nintendo points out, Alpex's comparison is misleading because it fails to keep the effective annual rates constant for purposes of both the mid-year calculation and the quarterly calculation. Therefore, I cannot conclude that Alpex's mid-year convention accurately approximates a quarterly payment schedule. I believe that it is fully within my discretion to accept the more straightforward, year-end calculation proposed by Nintendo.

To summarize, I find that an award of prejudgment interest is warranted. Such interest shall be calculated on an after-tax, year-end basis at the commercial paper rate. According to the testimony of Mr. Sherwin, which I accept as authoritative, prejudgment interest on the jury's verdict through August 1, 1994 is $37,220,311. Interest on the jury's verdict from August 1, 1994 to the date of judgment will be addressed by Mr. Sherwin in the supplemental affidavit ordered herein. In addition, Alpex is entitled to interest on the 6% royalties payable on Nintendo's infringing revenues between December 1, 1992 and May 31, 1994. Within 21 days of the date hereof, Nintendo shall submit a supplemental affidavit by Mr. Sherman, or such other qualified expert, specifying prejudgment interest (calculated in accordance with the ruling herein) on royalties payable on its infringing revenues between December 1992 and May 1994.

CONCLUSION

For the reasons set forth above, the court denies Nintendo's motions for judgment of non-infringement and judgment of invalidity as a matter of law; the court denies Nintendo's motion for a new trial on liability; and the court also denies Nintendo's motion for a remittitur, new trial, or judgment as a matter of law with respect to damages. Alpex's motion for entry of judgment is granted in part and denied in part. Specifically, the court grants Alpex's motion for an accounting with respect to Nintendo's infringing revenues between December 1, 1992 and May 31, 1994. Judgment shall be entered in an amount that, in addition to the jury's award, includes 6% of Nintendo's infringing revenues during the period between December 1, 1992 and May 31, 1994. The court also grants Alpex's motion for pre-judgment interest, directing that such interest be calculated on year-end royalty payments, and on an after-tax basis, using the commercial paper rate, as proposed by Nintendo. Alpex shall also be entitled to prejudgment interest on the 6% of Nintendo's infringing revenues between December 1, 1992 and May 31, 1994, to be determined through the aforementioned accounting. Finally, the court denies Alpex's motion for a 50% enhancement of the jury's verdict. Within 21 days of the date hereof, Nintendo shall submit (1) an accounting of its infringing revenues between December 1, 1992 and May 31, 1994, and (2) a supplemental affidavit specifying the prejudgment interest on the 6% royalty payments due on these additional revenues. Within one week of Nintendo's submission, the parties shall submit a joint proposed judgment in accordance with the court's ruling herein. In the event that the parties fail to agree upon a joint proposed judgment, they shall each submit a proposed judgment for the court's consideration.

SO ORDERED.

U.S. Court of Appeals
Federal Circuit

In re Deuel

No. 94-1202

Decided March 28, 1995

PATENTS

1. Patentability/Validity — Obviousness — Relevant prior art — Particular inventions (§115.0903.03)

Existence of general method of isolating cDNA or DNA molecules is essentially irrelevant to question of whether specific molecules themselves would have been obvious, in absence of other prior art that suggests claimed DNAs, nor does fact that general process can be conceived in advance for preparing undefined compound mean that claimed specific compound was precisely envisioned and therefore obvious; Board of Patent Appeals and Interferences thus erred by rejecting claims for isolated and purified

ith respect to Nintendo's infringing
ies between December 1, 1992 and
1, 1994. Judgment shall be entered in
iount that, in addition to the jury's
, includes 6% of Nintendo's infringing
ies during the period between Decem-
1992 and May 31, 1994. The court
ants Alpex's motion for pre-judgment
it, directing that such interest be calcu-
on year-end royalty payments, and on
er-tax basis, using the commercial pa-
te, as proposed by Nintendo. Alpex
lso be entitled to prejudgment interest
6% of Nintendo's infringing revenues
en December 1, 1992 and May 31,
to be determined through the afore-
ned accounting. Finally, the court de-
lpex's motion for a 50% enhancement
jury's verdict. Within 21 days of the
ereof, Nintendo shall submit (1) an
iting of its infringing revenues be-
December 1, 1992 and May 31, 1994,
2) a supplemental affidavit specifying
ejudgment interest on the 6% royalty
nts due on these additional revenues.
n one week of Nintendo's submission,
arties shall submit a joint proposed
ient in accordance with the court's rul-
rein. In the event that the parties fail to
upon a joint proposed judgment, they
each submit a proposed judgment for
urt's consideration.
ORDERED.

U.S. Court of Appeals
Federal Circuit

In re Deuel

No. 94-1202

Decided March 28, 1995

NTS

tentability/Validity — Obviousness — Relevant prior art — Particular inventions (§115.0903.03)

stence of general method of isolating
or DNA molecules is essentially irrel-
to question of whether specific mole-
themselves would have been obvious, in
ce of other prior art that suggests
ed DNAs, nor does fact that general
ss can be conceived in advance for pre-
undefined compound mean that
ed specific compound was precisely en-
ed and therefore obvious; Board of Pat
ppeals and Interferences thus erred by
ing claims for isolated and purified

DNA and cDNA molecules encoding heparin-binding growth factors based upon alleged obviousness of method of making molecules, since applied references do not teach or suggest claimed cDNA molecules.

Appeal from the U.S. Patent and Trademark Office, Board of Patent Appeals and Interferences; 33 USPQ2d 1445.

Application for patent filed by Thomas F. Deuel, Yue-Sheng Li, Ned R. Siegel, and Peter G. Milner, serial no. 07/542,232). From decision of the Board of Patent Appeals and Interferences affirming examiner's final rejection of claims, applicants appeal. Reversed.

G. Harley Blosser and Donald G. Leavit, of Senniger, Powers, Leavitt & Roedel, St. Louis, Mo., for appellants.

Donald S. Chisum, of Morrison & Foerster, Seattle, Wash.; Debra A. Shetka, of Morrison & Foerster, Palo Alto, Calif.; and Robert P. Blackburn, Emeryville, Calif., for amicus curiae The Biotechnology Industry Association and The Bay Area Bioscience Center.

Teddy S. Gron, acting associate solicitor, Albin F. Drost, acting solicitor, and Nancy J. Linck, office of the solicitor, Arlington, Va., for appellee.

Before Archer, chief judge, Nies and Lourie, circuit judges.

Lourie, J.

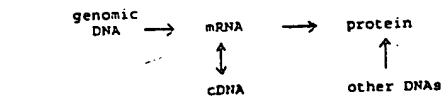
Thomas F. Deuel, Yue-Sheng Li, Ned R. Siegel, and Peter G. Milner (collectively "Deuel") appeal from the November 30, 1993 decision of the U.S. Patent and Trademark Office Board of Patent Appeals and Interferences affirming the examiner's final rejection of claims 4-7 of application Serial No. 07/542,232, entitled "Heparin-Binding Growth Factor," as unpatentable on the ground of obviousness under 35 U.S.C. § 103 (1988). *Ex parte Deuel*, 33 USPQ2d 1445 (Bd. Pat. App. Int. 1993). Because the Board erred in concluding that Deuel's claims 5 and 7 directed to specific cDNA molecules would have been obvious in light of the applied references, and no other basis exists in the record to support the rejection with respect to claims 4 and 6 generically covering all possible DNA molecules coding for the disclosed proteins, we reverse.

BACKGROUND

The claimed invention relates to isolated and purified DNA and cDNA molecules encoding heparin-binding growth factors ("HBGFs").[1] HBGFs are proteins that stimulate mitogenic activity (cell division) and thus facilitate the repair or replacement of damaged or diseased tissue. DNA (deoxyribonucleic acid) is a generic term which encompasses an enormous number of complex macromolecules made up of nucleotide units. DNAs consist of four different nucleotides containing the nitrogenous bases adenine, guanine, cytosine, and thymine. A sequential grouping of three such nucleotides (a "codon") codes for one amino acid. A DNA's sequence of codons thus determines the sequence of amino acids assembled during protein synthesis. Since there are 64 possible codons, but only 20 natural amino acids, most amino acids are coded for by more than one codon. This is referred to as the "redundancy" or "degeneracy" of the genetic code.

DNA functions as a blueprint of an organism's genetic information. It is the major component of genes, which are located on chromosomes in the cell nucleus. Only a small part of chromosomal DNA encodes functional proteins.

Messenger ribonucleic acid ("mRNA") is a similar molecule that is made or transcribed from DNA as part of the process of protein synthesis. Complementary DNA ("cDNA") is a complementary copy ("clone") of mRNA, made in the laboratory by reverse transcription of mRNA. Like mRNA, cDNA contains only the protein-encoding regions of DNA. Thus, once a cDNA's nucleotide sequence is known, the amino acid sequence of the protein for which it codes may be predicted using the genetic code relationship between codons and amino acids. The reverse is not true, however, due to the degeneracy of the code. Many other DNAs may code for a particular protein. The functional relationships between DNA, mRNA, cDNA, and a protein may conveniently be expressed as follows:



Collections ("libraries") of DNA and cDNA molecules derived from various spe-

[1] For a more extensive discussion of recombinant DNA technology, *see In re O'Farrell*, 853 F.2d 894, 895-99, 7 USPQ2d 1673, 1674-77 (Fed. Cir. 1988); *Amgen Inc. v. Chugai Pharmaceutical Co.*, 927 F.2d 1200, 18 USPQ2d 1016 (Fed. Cir.), *cert. denied*, 502 U.S. 856 (1991).

cies may be constructed in the laboratory or obtained from commercial sources. Complementary DNA libraries contain a mixture of cDNA clones reverse-transcribed from the mRNAs found in a specific tissue source. Complementary DNA libraries are tissue--specific because proteins and their corresponding mRNAs are only made ("expressed") in specific tissues, depending upon the protein. Genomic DNA ("gDNA") libraries, by contrast, theoretically contain all of a species' chromosomal DNA. The molecules present in cDNA and DNA libraries may be of unknown function and chemical structure, and the proteins which they encode may be unknown. However, one may attempt to retrieve molecules of interest from cDNA or gDNA libraries by screening such libraries with a gene probe, which is a synthetic radiolabelled nucleic acid sequence designed to bond ("hybridize") with a target complementary base sequence. Such "gene cloning" techniques thus exploit the fact that the bases in DNA always hybridize in complementary pairs: adenine bonds with thymine and guanine bonds with cytosine. A gene probe for potentially isolating DNA or cDNA encoding a protein may be designed once the protein's amino acid sequence, or a portion thereof, is known.

As disclosed in Deuel's patent application, Deuel isolated and purified HBGF from bovine uterine tissue, found that it exhibited mitogenic activity, and determined the first 25 amino acids of the protein's N-terminal sequence.[2] Deuel then isolated a cDNA molecule encoding bovine uterine HBGF by screening a bovine uterine cDNA library with an oligonucleotide probe designed using the experimentally determined N-terminal sequence of the HBGF. Deuel purified and sequenced the cDNA molecule, which was found to consist of a sequence of 1196 nucleotide base pairs. From the cDNA's nucleotide sequence, Deuel then predicted the complete amino acid sequence of bovine uterine HBGF disclosed in Deuel's application.

Deuel also isolated a cDNA molecule encoding human placental HBGF by screening a human placental cDNA library using the isolated bovine uterine cDNA clone as a probe. Deuel purified and sequenced the human placental cDNA clone, which was found to consist of a sequence of 961 nucleotide base pairs. From the nucleotide sequence of the cDNA molecule encoding human placental HBGF, Deuel predicted the complete amino acid sequence of human placental HBGF disclosed in Deuel's application. The predicted human placental and bovine uterine HBGFs each have 168 amino acids and calculated molecular weights of 18.9 kD. Of the 168 amino acids present in the two HBGFs discovered by Deuel, 163 are identical. Deuel's application does not describe the chemical structure of, or state how to isolate and purify, any DNA or cDNA molecule except the disclosed human placental and bovine uterine cDNAs, which are the subject of claims 5 and 7.

Claims 4-7 on appeal are all independent claims and read, in relevant part, as follows:

4. A purified and isolated DNA sequence consisting of a sequence encoding human heparin binding growth factor of 168 amino acids having the following amino acid sequence:

Met Gln Ala ... [remainder of 168 amino acid sequence].

5. The purified and isolated cDNA of human heparin-binding growth factor having the following nucleotide sequence:

GTCAAAGGCA ... [remainder of 961 nucleotide sequence].

6. A purified and isolated DNA sequence consisting of a sequence encoding bovine heparin binding growth factor of 168 amino acids having the following amino acid sequence:

Met Gln Thr ... [remainder of 168 amino acid sequence].

7. The purified and isolated cDNA of bovine heparin-binding growth factor having the following nucleotide sequence:

GAGTGGAGAG ... [remainder of 1196 nucleotide sequence].

Claims 4 and 6 generically encompass *all* isolated/purified DNA sequences (natural and synthetic) encoding human and bovine HBGFs, despite the fact that Deuel's application does not describe the chemical structure of, or tell how to obtain, any DNA or cDNA except the two disclosed cDNA molecules. Because of the redundancy of the genetic code, claims 4 and 6 each encompass an enormous number of DNA molecules, including the isolated/purified chromosomal DNAs encoding the human and bovine proteins. Claims 5 and 7, on the other hand, are directed to the specifically disclosed cDNA molecules encoding human and bovine HBGFs, respectively.

During prosecution, the examiner rejected claims 4-7 under 35 U.S.C. § 103 as unpatentable over the combined teachings of Boh-

[2] Deuel determined that the N-terminal sequence of bovine uterus HBGF is Gly - Lys - Lys - Glu - Lys - Pro - Glu - Lys - Lys - Val - Lys - Lys - Ser - Asp - Cys - Gly - Glu - Trp - Gln - Trp - Ser - Val - Cys - Val - Pro.

f the cDNA molecule encoding hu-
ental HBGF, Deuel predicted the
amino acid sequence of human
HBGF disclosed in Deuel's appli-
he predicted human placental and
erine HBGFs each have 168 amino
l calculated molecular weights of
Of the 168 amino acids present in
BGFs discovered by Deuel, 163 are

Deuel's application does not de-
chemical structure of, or state how
and purify, any DNA or cDNA
except the disclosed human placen-
vine uterine cDNAs, which are the
f claims 5 and 7.

4-7 on appeal are all independent
d read, in relevant part, as follows:

purified and isolated DNA se-
consisting of a sequence encoding
heparin binding growth factor of
ino acids having the following ami-
l sequence:
Gln Ala ... [remainder of 168
acid sequence].

he purified and isolated cDNA of
heparin-binding growth factor hav-
following nucleotide sequence:
AAAGGCA ... [remainder of
cleotide sequence].

purified and isolated DNA se-
consisting of a sequence encoding
heparin binding growth factor of
ino acids having the following ami-
l sequence:
Gln Thr ... [remainder of 168
acid sequence].

he purified and isolated cDNA of
heparin-binding growth factor hav-
following nucleotide sequence:
TGGAGAG ... [remainder of
ucleotide sequence].

4 and 6 generically encompass *all* purified DNA sequences (natural netic) encoding human and bovine despite the fact that Deuel's appli-es not describe the chemical struc-r tell how to obtain, any DNA or cept the two disclosed cDNA mole-cause of the redundancy of the de, claims 4 and 6 each encompass nous number of DNA molecules, the isolated/purified chromosomal coding the human and bovine pro-ims 5 and 7, on the other hand, are to the specifically disclosed cDNA s encoding human and bovine respectively.

prosecution, the examiner rejected 7 under 35 U.S.C. § 103 as unpa-ver the combined teachings of Boh-

len[3] and Maniatis.[4] The Bohlen reference discloses a group of protein growth factors designated as heparin-binding brain mitogens ("HBBMs") useful in treating burns and promoting the formation, maintenance, and repair of tissue, particularly neural tissue. Bohlen isolated three such HBBMs from human and bovine brain tissue. These proteins have respective molecular weights of 15 kD, 16 kD, and 18 kD. Bohlen determined the first 19 amino acids of the proteins' N-terminal sequences, which were found to be identical for human and bovine HBBMs.[5] Bohlen teaches that HBBMs are brain-specific, and suggests that the proteins may be homologous between species. The reference provides no teachings concerning DNA or cDNA coding for HBBMs.

Maniatis describes a method of isolating DNAs or cDNAs by screening a DNA or cDNA library with a gene probe. The reference outlines a general technique for cloning a gene; it does not describe how to isolate a particular DNA or cDNA molecule. Maniatis does not discuss certain steps necessary to isolate a target cDNA, *e.g.*, selecting a tissue-specific cDNA library containing a target cDNA and designing an oligonucleotide probe that will hybridize with the target cDNA.

The examiner asserted that, given Bohlen's disclosure of a heparin-binding protein and its N-terminal sequence and Maniatis's gene cloning method, it would have been *prima facie* obvious to one of ordinary skill in the art at the time of the invention to clone a gene for HBGF.[6] According to the examiner, Bohlen's published N-terminal sequence would have motivated a person of ordinary skill in the art to clone such a gene because cloning the gene would allow recombinant production of HBGF, a useful protein. The examiner reasoned that a person of ordinary skill in the art could have designed a gene probe based on Bohlen's disclosed N-terminal sequence, then screened a DNA library in accordance with Maniatis's gene cloning method to isolate a gene encoding an HBGF. The examiner did not distinguish between claims 4 and 6 generically directed to all DNA sequences encoding human and bovine HBGFs and claims 5 and 7 reciting particular cDNAs.

In reply, Deuel argued, *inter alia*, that Bohlen teaches away from the claimed cDNA molecules because Bohlen suggests that HBBMs are brain-specific and, thus, a person of ordinary skill in the art would not have tried to isolate corresponding cDNA clones from human placental and bovine uterine cDNA libraries. The examiner made the rejection final, however, asserting that

> [t]he starting materials are not relevant in this case, because it was well known in the art at the time the invention was made that proteins, especially the general class of heparin binding proteins, are highly homologous between species and tissue type. It would have been entirely obvious to attempt to isolate a known protein from different tissue types and even different species.

No prior art was cited to support the proposition that it would have been obvious to screen human placental and bovine uterine cDNA libraries for the claimed cDNA clones. Presumably, the examiner was relying on Bohlen's suggestion that HBBMs may be homologous between species, although the examiner did not explain how homology between species suggests homology between tissue types.

The Board affirmed the examiner's final rejection. In its opening remarks, the Board noted that it is "constantly advised by the patent examiners, who are highly skilled in this art, that cloning procedures are routine in the art." According to the Board, "the examiners urge that when the sequence of a protein is placed into the public domain, the gene is also placed into the public domain because of the routine nature of cloning techniques." Addressing the rejection at issue, the Board determined that Bohlen's disclosure of the existence and isolation of HBBM, a functional protein, would also advise a person of ordinary skill in the art that a gene exists encoding HBBM. The Board found that a person of ordinary skill in the art would have been motivated to isolate such a gene because the protein has useful mitogenic properties, and isolating the gene for HBBM would permit large quantities of the protein to be produced for study and possible commercial use. Like the examiner,

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[3] European Patent Application No. 0326075, naming Peter Bohlen as inventor, published August 2, 1989.

[4] Maniatis et al., *Molecular Cloning: A Laboratory Manual*, "Screening Bacteriophage [lambda] Libraries for Specific DNA Sequences by Recombination in *Escherichia coli*," Cold Spring Harbor Laboratory, New York, 1982, pp. 353-361.

[5] Bohlen's disclosed N-terminal sequence for human and bovine HBBMs is Gly - Lys - Lys - Glu - Lys - Pro - Glu - Lys - Lys - Val - Lys - Lys - Ser - Asp - Cys - Gly - Glu - Trp - Gln. This sequence matches the first 19 amino acids of Deuel's disclosed N-terminal sequence.

[6] The examiner and the Board apparently used the term "gene" to refer both to natural (chromosomal) DNA and synthetic cDNA. We will use the several terms as appropriate.

the Board asserted, without explanation, that HBBMs are the same as HBGFs and that the genes encoding these proteins are identical. The Board concluded that "the Bohlen reference would have suggested to those of ordinary skill in this art that they should make the gene, and the Maniatis reference would have taught a technique for 'making' the gene with a reasonable expectation of success." Responding to Deuel's argument that the claimed cDNA clones were isolated from human placental and bovine uterine cDNA libraries, whereas the combined teachings of Bohlen and Maniatis would only have suggested screening a brain tissue cDNA library, the Board stated that "the claims before us are directed to the product and not the method of isolation. Appellants have not shown that the claimed DNA was not present in and could not have been readily isolated from the brain tissue utilized by Bohlen." Deuel now appeals.[1]

DISCUSSION

Obviousness is a question of law, which we review *de novo*, though factual findings underlying the Board's obviousness determination are reviewed for clear error. *In re Vaeck*, 947 F.2d 488, 493, 20 USPQ2d 1438, 1442 (Fed. Cir. 1991); *In re Woodruff*, 919 F.2d 1575, 1577, 16 USPQ2d 1934, 1935 (Fed. Cir. 1990). The examiner bears the burden of establishing a *prima facie* case of obviousness. *In re Rijckaert*, 9 F.3d 1531, 1532, 28 USPQ2d 1955, 1956 (Fed. Cir. 1993); *In re Oetiker*, 977 F.2d 1443, 1445, 24 USPQ2d 1443, 1444 (Fed. Cir. 1992). Only if this burden is met does the burden of coming forward with rebuttal argument or evidence shift to the applicant. *Rijckaert*, 9 F.3d at 1532, 28 USPQ2d at 1956. When the references cited by the examiner fail to establish a *prima facie* case of obviousness, the rejection is improper and will be overturned. *In re Fine*, 837 F.2d 1071, 1074, 5 USPQ2d 1596, 1598 (Fed. Cir. 1988).

On appeal, Deuel challenges the Board's determination that the applied references establish a *prima facie* case of obviousness. In response, the PTO maintains that the claimed invention would have been *prima facie* obvious over the combined teachings of Bohlen and Maniatis. Thus, the appeal raises the important question whether the combination of a prior art reference teaching a method of gene cloning, together with a reference disclosing a partial amino acid sequence of a protein, may render DNA and cDNA molecules encoding the protein *prima facie* obvious under § 103.

[1] Deuel argues that the PTO failed to follow the proper legal standard in determining that the claimed cDNA molecules would have been *prima facie* obvious despite the lack of structurally similar compounds in the prior art. Deuel argues that the PTO has not cited a reference teaching cDNA molecules, but instead has improperly rejected the claims based on the alleged obviousness of a method of making the molecules. We agree.

Because Deuel claims new chemical entities in structural terms, a *prima facie* case of unpatentability requires that the teachings of the prior art suggest *the claimed compounds* to a person of ordinary skill in the art. Normally a *prima facie* case of obviousness is based upon structural similarity, *i.e.*, an established structural relationship between a prior art compound and the claimed compound. Structural relationships may provide the requisite motivation or suggestion to modify known compounds to obtain new compounds. For example, a prior art compound may suggest its homologs because homologs often have similar properties and therefore chemists of ordinary skill would ordinarily contemplate making them to try to obtain compounds with improved properties. Similarly, a known compound may suggest its analogs or isomers, either geometric isomers (cis v. trans) or position isomers (*e.g.*, ortho v. para).

In all of these cases, however, the prior art teaches a specific, structurally-definable compound and the question becomes whether the prior art would have suggested making the specific molecular modifications necessary to achieve the claimed invention. *See In re Jones*, 958 F.2d 347, 351, 21 USPQ2d 1941, 1944 (Fed. Cir. 1992); *In re Dillon*, 919 F.2d 688, 692, 16 USPQ2d 1897, 1901 (Fed. Cir. 1990) (en banc) ("structural similarity between claimed and prior art subject matter, ... where the prior art gives reason or motivation to make the claimed compositions, creates a *prima facie* case of obviousness"), *cert. denied*, 500 U.S. 904 (1991); *In re Grabiak*, 769 F.2d 729, 731-32, 226 USPQ 870, 872 (Fed. Cir. 1985) ("[I]n the case before us there must be adequate support in the prior art for the [prior art] ester [claimed] thioester change in structure, in order to complete the PTO's *prima facie*

[1] Deuel is supported in its appeal by an *amicus curiae* brief submitted by the Biotechnology Industry Organization and the Bay Area Science Center. Amici urge that, contrary to controlling precedent, the PTO has unlawfully adopted a *per se* rule that a gene is *prima facie* obvious when at least part of the amino acid sequence of the protein encoded by the gene is known in the prior art.

vious over the combined teachings of
ınd Maniatis. Thus, the appeal raises
ortant question whether the combina-
prior art reference teaching a meth-
ne cloning, together with a reference
ıg a partial amino acid sequence of a
may render DNA and cDNA mole-
coding the protein *prima facie* obvi-
:r § 103.

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ıe proper legal standard in determin-
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he prior art for the [prior art] ester/
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case and shift the burden of going forward to the applicant."); *In re Lalu*, 747 F.2d 703, 705, 223 USPQ 1257, 1258 (Fed. Cir. 1984) ("The prior art must provide one of ordinary skill in the art the motivation to make the proposed molecular modifications needed to arrive at the claimed compound.").

Here, the prior art does not disclose any relevant cDNA molecules, let alone close relatives of the specific, structurally-defined cDNA molecules of claims 5 and 7 that might render them obvious. Maniatis suggests an allegedly obvious process for trying to isolate cDNA molecules, but that, as we will indicate below, does not fill the gap regarding the subject matter of claims 5 and 7. Further, while the general idea of the claimed molecules, their function, and their general chemical nature may have been obvious from Bohlen's teachings, and the knowledge that some gene existed may have been clear, the precise cDNA molecules of claims 5 and 7 would not have been obvious over the Bohlen reference because Bohlen teaches proteins, not the claimed or closely related cDNA molecules. The redundancy of the genetic code precluded contemplation of or focus on the specific cDNA molecules of claims 5 and 7. Thus, one could not have conceived the subject matter of claims 5 and 7 based on the teachings in the cited prior art because, until the claimed molecules were actually isolated and purified, it would have been highly unlikely for one of ordinary skill in the art to contemplate what was ultimately obtained. What cannot be contemplated or conceived cannot be obvious.

The PTO's theory that one might have been motivated to try to do what Deuel in fact accomplished amounts to speculation and an impermissible hindsight reconstruction of the claimed invention. It also ignores the fact that claims 5 and 7 are limited to specific compounds, and any motivation that existed was a general one, to try to obtain a gene that was yet undefined and may have constituted many forms. A general motivation to search for some gene that exists does not necessarily make obvious a specifically-defined gene that is subsequently obtained as a result of that search. More is needed and it is not found here.

The genetic code relationship between proteins and nucleic acids does not overcome the deficiencies of the cited references. A prior art disclosure of the amino acid sequence of a protein does not necessarily render particular DNA molecules encoding the protein obvious because the redundancy of the genetic code permits one to hypothesize an enormous number of DNA sequences coding for the protein. No particular one of these DNAs can be obvious unless there is something in the prior art to lead to the particular DNA and indicate that it should be prepared. We recently held in *In re Baird*, 16 F.3d 380, 29 USPQ2d 1550 (Fed. Cir. 1994), that a broad genus does not necessarily render obvious each compound within its scope. Similarly, knowledge of a protein does not give one a conception of a particular DNA encoding it. Thus, *a fortiori*, Bohlen's disclosure of the N-terminal portion of a protein, which the PTO urges is the same as HBGF, would not have suggested the particular cDNA molecules defined by claims 5 and 7. This is so even though one skilled in the art knew that some DNA, albeit not in purified and isolated form, did exist. The compounds of claims 5 and 7 are specific compounds not suggested by the prior art. A different result might pertain, however, if there were prior art, *e.g.*, a protein of sufficiently small size and simplicity, so that lacking redundancy, each possible DNA would be obvious over the protein. *See In re Petering*, 301 F.2d 676 (CCPA 1962) (prior art reference disclosing limited genus of 20 compounds rendered every species within the genus unpatentable). That is not the case here.

The PTO's focus on known methods for potentially isolating the claimed DNA molecules is also misplaced because the claims at issue define compounds, not methods. *See In re Bell*, 991 F.2d 781, 785, 26 USPQ2d 1529, 1532 (Fed. Cir. 1993). In *Bell*, the PTO asserted a rejection based upon the combination of a primary reference disclosing a protein (*and its complete amino acid sequence*) with a secondary reference describing a general method of gene cloning. We reversed the rejection, holding in part that "[t]he PTO's focus on Bell's method is misplaced. Bell does not claim a method. Bell claims compositions, and the issue is the obviousness of the claimed compositions, not of the method by which they are made." *Id.*

We today reaffirm the principle, stated in *Bell*, that the existence of a general method of isolating cDNA or DNA molecules is essentially irrelevant to the question whether the specific molecules themselves would have been obvious, in the absence of other prior art that suggests the claimed DNAs. A prior art disclosure of a process *reciting a particular compound* or obvious variant thereof as a product of the process is, of course, another matter, raising issues of anticipation under 35 U.S.C. § 102 as well as obviousness under § 103. Moreover, where there is prior art that suggests a claimed compound, the existence, or lack thereof, of an enabling process for making that compound is surely a factor in

any patentability determination. *See In re Brown*, 329 F.2d 1006, 141 USPQ 245 (CCPA 1964) (reversing rejection for lack of an enabling method of making the claimed compound). There must, however, still be prior art that suggests the claimed compound in order for a *prima facie* case of obviousness to be made out; as we have already indicated, that prior art was lacking here with respect to claims 5 and 7. Thus, even if, as the examiner stated, the existence of general cloning techniques, coupled with knowledge of a protein's structure, might have provided motivation to prepare *a* cDNA or made it obvious to prepare *a* cDNA, that does not necessarily make obvious a particular claimed cDNA. "Obvious to try" has long been held not to constitute obviousness. *In re O'Farrell*, 853 F.2d 894, 903, 7 USPQ2d 1673, 1680-81 (Fed. Cir. 1988). A general incentive does not make obvious a particular result, nor does the existence of techniques by which those efforts can be carried out. Thus, Maniatis's teachings, even in combination with Bohlen, fail to suggest the claimed invention.

The PTO argues that a compound may be defined by its process of preparation and therefore that a conceived process for making or isolating it provides a definition for it and can render it obvious. It cites *Amgen Inc. v. Chugai Pharmaceutical Co.*, 927 F.2d 1200, 18 USPQ2d 1016 (Fed. Cir.), *cert. denied*, 502 U.S. 856 (1991), for that proposition. We disagree. The fact that one can conceive a general process in advance for preparing an *undefined* compound does not mean that a claimed *specific* compound was precisely envisioned and therefore obvious. A substance may indeed be defined by its process of preparation. That occurs, however, when it has already been prepared by that process and one therefore knows that the result of that process is the stated compound. The process is part of the definition of the compound. But that is not possible in advance, especially when the hypothetical process is only a general one. Thus, a conceived method of preparing some undefined DNA does not define it with the precision necessary to render it obvious over the protein it encodes. We did not state otherwise in *Amgen*. *See Amgen*, 927 F.2d at 1206-9, 18 USPQ2d at 1021-23 (isolated/purified human gene held nonobvious; no conception of gene without envisioning its precise identity despite conception of general process of preparation).

We conclude that, because the applied references do not teach or suggest the claimed cDNA molecules, the final rejection of claims 5 and 7 must be reversed. *See also Bell*, 991 F.2d at 784-85, 26 USPQ2d at 1531-32 (human DNA sequences encoding IGF proteins nonobvious over asserted combination of references showing gene cloning method and complete amino acid sequences of IGFs).

Claims 4 and 6 are of a different scope than claims 5 and 7. As is conceded by Deuel, they generically encompass all DNA sequences encoding human and bovine HBGFs. Written in such a result-oriented form, claims 4 and 6 are thus tantamount to the general idea of all genes encoding the protein, all solutions to the problem. Such an idea might have been obvious from the *complete* amino acid sequence of the protein, coupled with knowledge of the genetic code, because this information may have enabled a person of ordinary skill in the art to envision the idea of, and, perhaps with the aid of a computer, even identify all members of the claimed genus. The Bohlen reference, however, only discloses a partial amino acid sequence, and thus it appears that, based on the above analysis, the claimed genus would not have been obvious over this prior art disclosure. We will therefore also reverse the final rejection of claims 4 and 6 because neither the Board nor the patent examiner articulated any separate reasons for holding these claims unpatentable apart from the grounds discussed above.

One further matter requires comment. Because Deuel's patent application does not describe how to obtain any DNA except the disclosed cDNA molecules, claims 4 and 6 may be considered to be inadequately supported by the disclosure of the application. *See generally Amgen Inc. v. Chugai Pharmaceutical Co.*, 927 F.2d 1200, 1212-14, 18 USPQ2d 1016, 1026-28 (Fed. Cir.) (generic DNA sequence claims held invalid under 35 U.S.C. § 112, first paragraph), *cert. denied*, 502 U.S. 856 (1991); *In re Fisher*, 427 F.2d 833, 839, 166 USPQ 18, 24 (CCPA 1970) (Section 112 "requires that the scope of the claims must bear a reasonable correlation to the scope of enablement provided by the specification to persons of ordinary skill in the art."). As this issue is not before us, however, we will not address whether claims 4 and 6 satisfy the enablement requirement of § 112, first paragraph, but will leave to the PTO the question whether any further rejection is appropriate.

We have considered the PTO's remaining arguments and find them not persuasive.

CONCLUSION

The Board's decision affirming the final rejection of claims 4-7 is reversed.

REVERSED

and instead render our decision on the merits. Fed. R. App. P. 42(b). The judgment below is affirmed.

Court of Appeals, Federal Circuit

In re Bell

No. 92-1375

Decided April 20, 1993

PATENTS

1. Patentability/Validity — Obviousness — In general (§115.0901)

Patentability/Validity — Obviousness — Relevant prior art — Particular inventions (§115.0903.03)

Established relationship in genetic code between nucleic acid and protein it encodes does not make gene prima facie obvious over its correspondent protein in same way that closely related homologs, analogs, and isomers in chemistry may create prima facie case, since there are vast number of nucleotide sequences that might code for specific protein due to "degeneracy" of genetic code; gene might be obvious over correspondent protein if latter is known amino acid sequence specified exclusively by "unique" codons, but claims in application for nucleic acid molecules containing human sequences coding for human insulin-like growth factors I and II (IGF) are not obvious in view of cited prior art disclosing amino acid sequences for IGF I and II, since cited art suggests nearly infinite number of sequences, but fails to suggest which of those are human nucleic acid sequences coding for IGF.

2. Patentability/Validity — Obviousness — Combining references (§115.0905)

Reference disclosing general method for isolating genes, in combination with prior art disclosing amino acid sequences for insulin-like growth factors I and II (IGF), does not render obvious application claims for nucleic acid molecules containing human sequences coding for human IGF I and II, since, absent some teaching or suggestion supporting combination, obviousness is not established by combining teachings of prior art to produce claimed invention, since reference in question teaches away from invention claimed in application by emphasizing importance of "unique" codons, and since reference thus cannot be held to "fairly suggest" that its teachings be combined with those of prior art, which discloses amino acid sequences lacking "unique" codons.

3. Patentability/Validity — Obviousness — In general (§115.0901)

Patent construction — Claims — Process (§125.1309)

Similarities between method by which applicants made claimed nucleic acid molecules, and method for isolating genes taught by prior art reference, do not render application claims obvious, since applicants claim compositions, rather than method of making them.

Appeal from the U.S. Patent and Trademark Office, Board of Patent Appeals and Interferences.

Patent application of Graeme I. Bell, Leslie B. Rall and James P. Merryweather, serial no. 07/065,673 ("preproinsulin-like growth factors I and II"). From decision affirming examiner's final rejection of claims 25-46, applicants appeal. Reversed.

Robert P. Blackburn, Emeryville, Calif. (Debra A. Shetka and Thomas E. Ciotti, of Morrison & Foerster, Palo Alto, Calif., and Donald S. Chisum, of Morrison & Foerster, Seattle, Wash., on brief), for appellant.

Teddy S. Gron, associate solicitor (Fred E. McKelvey, solicitor, on brief; John W. Dewhirst, Lee E. Barrett, Richard E. Schafer, and Albin F. Drost, of counsel), for PTO.

Before Rich, Lourie, and Schall, circuit judges.

Lourie, J.

Applicants Graeme I. Bell, Leslie B. Rall, and James P. Merryweather (Bell) appeal from the March 10, 1992 decision of the U.S. Patent and Trademark Office (PTO) Board of Patent Appeals and Interferences, Appeal No. 91-1124, affirming the examiner's final rejection of claims 25-46 of application Serial No. 065,673, entitled "Preproinsulin-Like Growth Factors I and II," as unpatentable on the ground of obviousness under 35 U.S.C. § 103 (1988). Because the Board erred in concluding that the claimed nucleic acid molecules would have been obvious in light of the cited prior art, we reverse.

BACKGROUND

The claims of the application at issue are directed to nucleic acid molecules (DNA and

RNA)[1] containing human sequences[2] which code for human insulin-like growth factors I and II (IGF), single chain serum proteins that play a role in the mediation of somatic cell growth following the administration of growth hormones.[3]

The relevant prior art consists of two publications by Rinderknecht[4] disclosing amino acid sequences for IGF-I and -II and U.S. Patent 4,394,443 to Weissman et al., entitled "Method for Cloning Genes." Weissman describes a general method for isolating a gene for which at least a short amino acid sequence of the encoded protein is known. The method involves preparing a nucleotide probe corresponding to the known amino acid sequence and using that probe to isolate the gene of interest. It teaches that it is advantageous to design a probe based on amino acids specified by unique codons.[5] The Weissman patent specifically describes the isolation of a gene which codes for human histocompatibility antigen, a protein unrelated to IGF. It describes the design of the probe employed, stating that it was based on amino acids specified by unique codons.

The examiner rejected the claims as obvious over the combined teachings of Rinderknecht and Weissman. She determined that it would have been obvious, "albeit tedious," from the teachings of Weissman to prepare probes based on the Rinderknecht amino acid sequences to obtain the claimed nucleic acid molecules. According to the examiner, "it is clear from [Weissman] that the ordinary artisan knows how to find the nucleic acid when the amino acid sequence is known" and that "the claimed sequences and hosts would have been readily determinable by and obvious to those of ordinary skill in the art at the time the invention was made."

The Board affirmed the examiner's rejection, holding that the examiner had established a *prima facie* case of obviousness for the claimed sequences "despite the lack of conventional indicia of obviousness, e.g., structural similarity between the DNA which codes for IGF-I and the amino acid sequence of the polypeptide which constitues [sic] IGF-I." Slip op. at 6. The Board reasoned that "although a protein and its DNA are not structurally similar, they are correspondently linked via the genetic code." *Id.* at 4 n.1. In view of Weissman, the Board concluded that there was no evidence "that one skilled in the art, knowing the amino acid sequences of the desired proteins, would not have been able to predictably clone the de-

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[1] A basic familiarity with recombinant DNA technology is presumed. For a general discussion, *see In re O'Farrell*, 853 F.2d 894, 895-99, 7 USPQ2d 1673, 1674-77 (Fed. Cir. 1988).

[2] Interchangeably referred to as "native" sequences and "genes."

[3] Claim 25 is conceded to be representative of the claims at issue:

A composition comprising nucleic acid molecules containing a human sequence encoding insulin-like growth factor (hIGF) substantially free of nucleic acid molecules not containing said hIGF sequence, wherein said hIGF sequence is selected from the group consisting of:

(a) 5'-GGA CCG GAG ACG CUC UGC GGG GCU GAG CUG GUG GAU GCU CUU CAG UUC GUG UGU GGA GAC AGG GGC UUU UAU UUC AAC AAG CCC ACA GGG UAU GGC UCC AGC AGU CGG AGG GCG CCU CAG ACA GGU AUC GUG GAU GAG UGC UGC UUC CGG AGC UGU GAU CUA AGG AGG CUG GAG AUG UAU UGC GCA CCC CUC AAG CCU GCC AAG UCA GCU-3', wherein U can also be T;

(b) 5'-GCU UAC CGC CCC AGU GAG ACC CUG UGC GGC GGG GAG CUG GUG GAC ACC CUC CAG UUC GUC UGU GGG GAC CGC GGC UUC UAC UUC AGC AGG CCC GCA AGC CGU GUG AGC CGU CGC AGC CGU GGC AUC GUU GAG GAG UGC UGU UUC CGC AGC UGU GAC CUG GCC CUC CUG GAG ACG UAC UGU GCU ACC CCC GCC AAG UCC GAG-3', wherein U can also be T;

(c) nucleic acid sequences complementary to (a) or (b); and

(d) fragments of (a), (b) or (c) that are at least 18 bases in length and which will selectively hybridize to human genomic DNA encoding hIGF.

The other rejected claims are apparently directed to cellular hosts transformed with the claimed nucleic acid sequences. Because their fate is dependent upon that of claim 25, neither appellant nor the Patent and Trademark Office have considered them separately and we will not do so either.

[4] Rinderknecht et al., *The Amino Acid Sequence of Human Insulin-like Growth Factor I and Its Structural Homology with Proinsulin*, 253 The Journal of Biological Chemistry 2769-76 (1978); Rinderknecht et al., *Primary Structure of Human Insulin-like Growth Factor II*, 89 FEB Letters 283-86 (May 1978).

[5] A sequence of three nucleotides, called a codon, codes for each of the twenty natural amino acids. Since there are twenty amino acids and sixty-four possible codons, most amino acids are specified by more than one codon. This is referred to as "degeneracy" in the genetic code. The term "unique" refers to an amino acid coded for by a single codon. *See Amgen Inc. v. Chugai Pharmaceutical Co.*, 927 F.2d 1200, 1207-08 n.4, 18 USPQ2d 1016, 1022 n.4 (Fed. Cir.), *cert. denied*, 112 S. Ct. 169 (1991).

sired DNA sequences without undue experimentation." *Id.* at 8.

The issue before us is whether the Board correctly determined that the amino acid sequence of a protein in conjunction with a reference indicating a general method of cloning renders the gene *prima facie* obvious.

DISCUSSION

We review an obviousness determination by the Board *de novo*. *In re Vaeck*, 947 F.2d 488, 493, 20 USPQ2d 1438, 1442 (Fed. Cir. 1991). Bell argues that the PTO has not shown how the prior art references, either alone or in combination, teach or suggest the claimed invention, and thus that it has failed to establish a *prima facie* case of obviousness.

We agree. The PTO bears the burden of establishing a case of *prima facie* obviousness. *In re Fine*, 837 F.2d 1071, 1074, 5 USPQ2d 1596, 1598 (Fed. Cir. 1988). "A prima facie case of obviousness is established when the teachings from the prior art itself would appear to have suggested the claimed subject matter to a person of ordinary skill in the art." *In re Rinehart*, 531 F.2d 1048, 1051, 189 USPQ 143, 147 (CCPA 1976).

The Board supported the examiner's view that the "correspondent link" between a gene and its encoded protein via the genetic code renders the gene obvious when the amino acid sequence is known. In effect, this amounts to a rejection based on the Rinderknecht references alone. Implicit in that conclusion is the proposition that, just as closely related homologs, analogs, and isomers in chemistry may create a *prima facie* case, *see In re Dillon*, 919 F.2d 688, 696, 16 USPQ2d 1897, 1904 (Fed. Cir. 1990) (*in banc*), *cert. denied*, 111 S. Ct. 1682 (1991), the established relationship in the genetic code between a nucleic acid and the protein it encodes also makes a gene *prima facie* obvious over its correspondent protein.

[1] We do not accept this proposition. It may be true that, knowing the structure of the protein, one can use the genetic code to hypothesize possible structures for the corresponding gene and that one thus has the potential for obtaining that gene. However, because of the degeneracy of the genetic code, there are a vast number of nucleotide sequences that might code for a specific protein. In the case of IGF, Bell has argued without contradiction that the Rinderknecht amino acid sequences could be coded for by more than 10^{36} different nucleotide sequences, only a few of which are the human sequences that Bell now claims. Therefore, given the nearly infinite number of possibilities suggested by the prior art, and the failure of the cited prior art to suggest which of those possibilities is the human sequence, the claimed sequences would not have been obvious.

Bell does not claim all of the 10^{36} nucleic acids that might potentially code for IGF. Neither does Bell claim all nucleic acids coding for a protein having the biological activity of IGF. Rather, Bell claims only the human nucleic acid sequences coding for IGF. Absent anything in the cited prior art suggesting which of the 10^{36} possible sequences suggested by Rinderknecht corresponds to the IGF gene, the PTO has not met its burden of establishing that the prior art would have suggested the claimed sequences.

This is not to say that a gene is never rendered obvious when the amino acid sequence of its coded protein is known. Bell concedes that in a case in which a known amino acid sequence is specified exclusively by unique codons, the gene might have been obvious. Such a case is not before us.[6] Here, where Rinderknecht suggests a vast number of possible nucleic acid sequences, we conclude that the claimed human sequences would not have been obvious.

[2] Combining Rinderknecht with Weissman does not fill the gap. Obviousness "'cannot be established by combining the teachings of the prior art to produce the claimed invention, absent some teaching or suggestion supporting the combination.'" *In re Fine*, 837 F.2d at 1075, 5 USPQ2d at 1598 (citing *ACS Hosp. Sys. v. Montefiore Hosp.*, 732 F.2d 1572, 1577, 221 USPQ 929, 933 (Fed. Cir. 1984)). What a reference teaches and whether it teaches toward or away from the claimed invention are questions of fact. *See Raytheon Co. v. Roper Corp.*, 724 F.2d 951, 960-61, 220 USPQ 592, 599-600 (Fed. Cir. 1983), *cert. denied*, 469 U.S. 835 [225 USPQ 232] (1984).

While Weissman discloses a general method for isolating genes, he appears to teach away from the claimed invention by emphasizing the importance of unique codons for the amino acids. Weissman suggests that it is generally advantageous to design a probe based on an amino acid sequence specified by unique codons, and also teaches that it is "counterproductive" to use a primer having

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[6] We also express no opinion concerning the reverse proposition, that knowledge of the structure of a DNA, e.g., a cDNA, might make a coded protein obvious.

more than 14-16 nucleotides unless the known amino acid sequence has 4-5 amino acids coded for by unique codons. Bell, in contrast, used a probe having 23 nucleotides based on a sequence of eight amino acids, none of which were unique. Weissman therefore tends to teach away from the claimed sequences since Rinderknecht shows that IGF-I has only a single amino acid with a unique codon and IGF-II has none.

The PTO, in urging us to affirm the Board, points to the suggestion in Weissman that the disclosed method can "easily" be applied to isolate genes for an array of proteins including peptide hormones. The PTO thus argues that in view of Weissman, a gene is rendered obvious once the amino acid sequence of its translated protein is known. We decline to afford that broad a scope to the teachings of Weissman. While "a reference must be considered not only for what it expressly teaches, but also for what it fairly suggests," *In re Burckel*, 592 F.2d 1175, 1179, 201 USPQ 67, 70 (CCPA 1979), we cannot say that Weissman "fairly suggests" that its teachings should be combined with those of Rinderknecht, since it nowhere suggests how to apply its teachings to amino acid sequences without unique codons.

We conclude that the Board clearly erred in determining that Weissman teaches toward, rather than away from, the claimed sequences. Therefore, the requisite teaching or suggestion to combine the teachings of the cited prior art references is absent, *see In re Fine*, 837 F.2d 1075, 5 USPQ2d at 1599, and the PTO has not established that the claimed sequences would have been obvious over the combination of Rinderknecht and Weissman.

[3] Finally, the PTO emphasizes the similarities between the method by which Bell made the claimed sequences and the method taught by Weissman. The PTO's focus on Bell's method is misplaced. Bell does not claim a method. Bell claims compositions, and the issue is the obviousness of the claimed compositions, not of the method by which they are made. *See In re Thorpe*, 777 F.2d 695, 697, 227 USPQ 964, 966 (Fed. Cir. 1985) ("The patentability of a product does not depend on its method of production.").

CONCLUSION

Because we conclude that the combination of prior art references does not render the claimed invention obvious, we reverse the Board's decision affirming the examiner's rejection of claims 25-46.

REVERSED

Court of Appeals, Fifth Circuit

Matrix Essentials Inc. v. Emporium Drug Mart Inc. of Lafayette

No. 91-4457

Decided April 19, 1993

TRADEMARKS AND UNFAIR TRADE PRACTICES

1. Infringement; conflicts between marks — Likelihood of confusion — In general (§335.0301)

Infringement; conflicts between marks — Passing off (§335.07)

Retail drug store's sale, without authorization from plaintiff, of plaintiff's cosmetic products under plaintiff's mark does not give rise to cause of action for trademark infringement under Lanham Act, even though plaintiff alleges that its products are not "genuine" because they are sold in defendant's store without professional cosmetologist consultation that is supposed to be available when consumers purchase plaintiff's products, since plaintiff has failed to show any consumer confusion or deception, nor does drug store's sale of plaintiff's products violate Lanham Act's Section 43(a), 15 USC 1125(a), since that section does not provide cause of action merely for unauthorized stocking and sale of manufacturer's products, absent more culpable conduct by seller.

Appeal from the U.S. District Court for the Western District of Louisiana, Scott, J.

Action by Matrix Essentials Inc. against Emporium Drug Mart Inc. of Lafayette, d/b/a Drug Emporium, for trademark infringement and unfair competition, in which Emporium filed counterclaim alleging antitrust violations. From federal district court's decision granting summary judgment dismissing both plaintiff's claims and counterclaim, parties cross-appeal. Affirmed.

Louis A. Colombo, of Baker & Hostetler, Cleveland, Ohio; Theresa M. Gallion, of Baker & Hostetler, Orlando, Fla.; and Louis Simon, II, of Laborde & Neuner, Lafayette, La., for plaintiff.

ratification can be accepted after expiration of the period for applying for renewal or filing an affidavit of continued use, because it is not a requirement of the statute.[5]

In this case, the renewal application filed October 23, 1989 contains a declaration, pursuant to Trademark Rule 2.20, signed by Anna Veronika Murray dba Murray Space Shoe Corporation, acknowledging co-ownership of the registration with Murray Space Shoe, Inc. and verifying the facts stated in the renewal application. While this document was filed too late to be accepted as a renewal application, it can be accepted for the purpose of ratifying the statements in the application which was filed on August 16, 1988 on behalf of the joint owners of the registration.

Accordingly, the petition is granted. The registration file will be forwarded to the Affidavit-Renewal Examiner, who is directed to consider the renewal application filed August 16, 1988 as being properly executed and filed by the registrant.

Court of Appeals, Federal Circuit

In re Jones

No. 91-1380

Decided February 28, 1992

PATENTS

1. Patentability/Validity — Obviousness — Relevant prior art — Particular inventions (§115.0903.03)

Claimed novel salt of acid commonly known as "dicamba" is not so closely related in structure to substituted ammonium salts disclosed in prior patent as to be prima facie obvious, since claimed salt is primary amine with ether linkage, whereas diethanolamino salt disclosed in reference patent is secondary amine without ether linkage, since claimed salt is plainly acyclic or linear, whereas morpholino salt, which is only substituted ammonium salt of dicamba with ether linkage disclosed in reference patent, is cyclic in structure, and since isopropylamino salt disclosed in reference patent is primary amine, but has iso-structure quite different from that of claimed salt.

2. Patentability/Validity — Obviousness — Relevant prior art — Particular inventions (§115.0903.03)

Claimed novel salt of acid commonly known as "dicamba" cannot be held prima facie obvious in view of salts disclosed in prior patent, even though claimed salt is member of genus of substituted ammonium salts broadly disclosed in reference patent, since reference discloses potentially infinite genus of "substituted ammonium salts" of dicamba, and lists several such salts, but does not specifically disclose salt claimed in application, and since claimed salt is not sufficiently similar to those disclosed in reference as to render it prima facie obvious.

3. Patentability/Validity — Obviousness — Combining references (§115.0905)

Contention that one skilled in herbicidal art would have been motivated to use, with acid commonly known as "dicamba," substituted ammonium salt such as that disclosed in two prior references does not warrant holding that claimed substituted ammonium salt of dicamba for use as herbicide is prima facie obvious, since there is no suggestion for combining disclosures of those references either in references themselves, which are directed to shampoo additives and production of morpholine, respectively, or in knowledge generally available to those skilled in art.

Appeal from the U.S. Patent and Trademark Office, Board of Patent Appeals and Interferences.

Patent application of Rita S. Jones, Michael T. Chirchirillo and Johnny L. Burns, serial no. 07/099,279 (the 2-(2'-aminoethoxy)-ethanol salt of dicamba). From decision upholding rejection of only claim in application, applicants appeal. Reversed.

Melvyn M. Kassenoff, East Hanover, N.J. (Gerald D. Sharkin and Richard E. Villa, East Hanover; Joanne M. Giesser, Palo Alto, Calif., with him on brief), for appellant.

Harris A. Pitlock, associate solicitor (Fred E. McKelvey, solicitor, with him on brief; Richard E. Schafer, of counsel), for appellee.

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[5] This is not inconsistent with Office practice under Section 1 of the Act. An application for registration by joint applicants under Section 1 which is signed by only one party is granted a filing date. Additional declarations by the other owner(s) verifying the facts stated in the application must be submitted during prosecution of the application, before the mark can be approved for publication.

Before Rich, Archer, and Clevenger, circuit judges.

Rich, J.

Rita S. Jones et al. (collectively Jones) appeal from the April 15, 1991 decision of the Patent and Trademark Office (PTO) Board of Patent Appeals and Interferences (Board), Appeal No. 90-1920, sustaining the rejection of claim 1, the only claim of application Ser. No. 07/099,279, titled "The 2-(2'-Aminoethoxy) — Ethanol Salt of Dicamba," as unpatentable under 35 USC 103. We conclude that the PTO has not presented a *prima facie* case of obviousness, and therefore *reverse*.

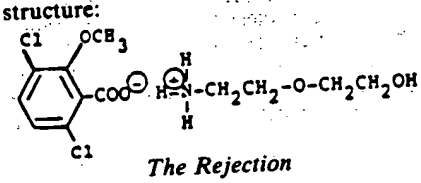
The Invention

The Claimed invention is a novel salt of 2-methoxy-3, 6-dichlorobenzoic acid, which acid is commonly referred to as "dicamba." A known herbicide, dicamba has typically been sold in the form of its known dimethylamine salt.

The sole claim of the application on appeal reads:

1. The 2-(2'-aminoethoxy) ethanol salt of dicamba.

The claimed salt has the following structure:



The Rejection

Claim 1 stands rejected as obvious in view of the combined teachings of the following references:

Richter, U.S. Patent No. 3,013,054, Dec. 12, 1961

Moyle et al., U.S. Patent No. 3,056,669, Oct. 2, 1962

Balassa, U.S. Patent No. 3,725,031, Apr. 3, 1973

Zorayan et al., 88 *Chem. Abstracts* No. 52300j, 1978

Wideman, 86 *Chem. Abstracts* No. 43711a, 1977

Richter, which all agree is the closest prior art, discloses dicamba in free acid, ester, and salt forms, for use as a herbicide. Among the salt forms disclosed are substituted ammonium salts, a genus which admittedly encompasses the claimed salt. Richter does not specifically disclose the claimed 2-(2'-aminoethoxy) ethanol salt, however. Most notably, Richter discloses (emphasis and bracketed word ours):

> Compositions in which X is substituted ammonium are amine salts of 2-methoxy-3, 6-dichlorobenzoic acid [dicamba] and are prepared by the addition of the free acid to various amines. Typical amines which can be used to prepare such amine salts are dimethylamine, trimethylamine, triethylamine, diethanolamine, triethanolamine, isopropylamine, morpholine, and the like. *The resulting products are, respectively, the dimethylamino, trimethylamino, triethylamino, diethanolamino, triethanolamino, isopropylamino, and morpholino salts of 2-methoxy-3, 6-dichlorobenzoic acid.*

Zorayan teaches the amine ($H_2N(CH_2CH_2O)_2H$) used to make the claimed salt, as well as the use of that amine in the preparation of surfactants for shampoos, bath preparations, and emulsifiers.

Wideman also teaches the amine disclosed in Zorayan.

The content of the remaining references is unnecessary to our decision.

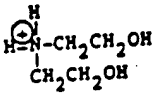
The Board upheld the examiner's rejection of claim 1 as obvious, finding that the claimed 2-2'-aminoethoxy) ethanol salt of dicamba and the diethanolamine salt of dicamba specifically disclosed by Richter were "closely related in structure," and that based upon the expectation that "compounds similar in structure will have similar properties," a *prima facie* case of obviousness had arisen. The Board found that Jones' rebuttal evidence (Rule 132 declarations and data reported in the specification) failed to "compare the claimed subject matter with the closest prior art," and accordingly did not serve to rebut the *prima facie* case. This appeal followed.

Analysis

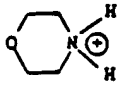
The Solicitor contends that the claimed salt falls within the genus of substituted amine salts of dicamba disclosed by Richter, and that, like Richter's genus, the claimed compound has herbicidal activity. Thus, the Solicitor urges, under the circumstances of this case, (1) the genus/species relationship and (2) the common utility of the claimed and prior art compounds support the Board's holding of *prima facie* obviousness. Moreover, the Solicitor adds, although the claimed compound is neither a homolog nor a position isomer of those salts specifically disclosed in Richter, it is structurally similar thereto, particularly the diethanolamino salt noted by the Board.

The question of "structural similarity" in chemical patent cases has generated a body of patent law unto itself.[1] Particular types or categories of structural similarity without more have, in past cases, given rise to *prima facie* obviousness; *see, e.g., In re Dillon*, 919 F.2d 688, 692-94, 16 USPQ2d 1897, 1900-02 (Fed. Cir. 1990) (tri-orthoesters and tetra-orthoesters), *cert. denied*, ——— U.S. ———, 111 S. Ct. 1682 (1991); *In re May*, 574 F.2d 1082, 197 USPQ 601 (CCPA 1978) (stereoisomers); *In re Wilder*, 563 F.2d 457, 195 USPQ 426 (CCPA 1977) (adjacent homologs and structural isomers); *In re Hoch*, 428 F.2d 1341, 166 USPQ 406 (CCPA 1970) (acid and ethyl ester). However, none of these types of structural similarity are involved here. And in any event, this court has previously stated that generalization is to be avoided insofar as specific structures are alleged to be *prima facie* obvious one from the other. *In re Grabiak*, 769 F.2d 729, 731, 226 USPQ 870, 872 (Fed. Cir. 1985).

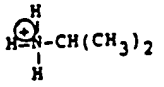
[1] On the basis of the record before us, we cannot sustain the Board's conclusion that the claimed salt and the diethanolamino salt disclosed by Richter are so "closely related in structure" as to render the former *prima facie* obvious in view of the latter. The claimed salt is a primary amine with an ether linkage. The diethanolamino salt disclosed by Richter is a secondary amine, without an ether linkage:



In addition, the only substituted ammonium salt of dicamba expressly disclosed by Richter having an ether linkage is the morpholino salt, which is *cyclic* in structure:



The claimed salt is, plainly, *a*cyclic; i.e., linear. Lastly, while the isopropylamino salt disclosed by Richter is a primary amine, as is the claimed salt, its iso- structure is quite different:



[2] The lack of close similarity of structure is not negated by the fact that the claimed salt is a member of Richter's broadly disclosed genus of substituted ammonium salts of dicamba. The Solicitor contends that "[t]he relative size of the genus disclosed by the prior art would not appear to be a controlling factor in determining whether a *prima facie* case of obviousness exists for a species encompassed within the described genus," citing *Merck & Co. v. Biocraft Labs., Inc.*, 874 F.2d 804, 806-09, 10 USPQ2d 1843, 1845-48 (Fed. Cir.), *cert. denied*, ——— U.S. ———, 110 S. Ct. 498 (1989). We decline to extract from *Merck* the rule that the Solicitor appears to suggest — that regardless of how broad, a disclosure of a chemical genus renders obvious any species that happens to fall within it. In *Merck*, at issue on appeal was whether claims to a composition of two diuretics, amiloride and hydrochlorothiazide, present in a particular "medically synergistic" weight ratio, would have been obvious in view of a specific prior art disclosure of amiloride in combination with hydrochlorothiazide, one of 1200 such combinations disclosed in the prior art reference. *Id.* at 806, 10 USPQ2d at 1845. Based on the facts before it, including evidence at trial that the experimentation needed to arrive at the claimed dosage was "nothing more than routine," *Id.* at 809, 10 USPQ2d at 1847, the court affirmed the trial court's determination of obviousness. In contrast, though Richter discloses the potentially infinite genus of "substituted ammonium salts" of dicamba, and lists several such salts, the salt claimed here is not specifically disclosed. Nor, as we have explained above, is the claimed salt sufficiently similar in structure to those specifically disclosed in Richter as to render it *prima facie* obvious. Every case, particularly those raising the issue of obviousness under section 103, must necessarily be decided upon its own facts.

[3] The Solicitor points out that, given the breadth of forms of dicamba (free acid, ester, or salt) disclosed by Richter as having herbicidal utility, one of ordinary skill in the art would appreciate that the dicamba group has significance with respect to imparting herbicidal activity to dicamba compounds. Thus, the Solicitor contends, one skilled in the art would have been motivated to use, with dicamba, substituted ammonium salts made from a known amine, such as the amine disclosed by Zorayan and Wideman, and would have expected such a salt to have herbicidal activity. Before the PTO may combine the disclosures of two or more prior art references in order to establish *prima facie* obviousness, there must be some sug-

[1] *See generally* Helmuth A. Wegner, "Prima Facie Obviousness of Chemical Compounds," 6 *Am. Pat. L. Assoc. O. J.* 271 (1978).

gestion for doing so, found either in the references themselves or in the knowledge generally available to one of ordinary skill in the art. *In re Fine*, 837 F.2d 1071, 1074, 5 USPQ2d 1596, 1598-99 (Fed. Cir. 1988). We see no such suggestion in Zorayan, which is directed to shampoo additives, nor in Wideman, which teaches that the amine used to make the claimed compound is a byproduct of the production of morpholine. Nor does the broad disclosure of Richter fill the gap, for the reasons discussed above.

Conspicuously missing from this record is any *evidence*, other than the PTO's speculation (if it be called evidence) that one of ordinary skill in the herbicidal art would have been motivated to make the modifications of the prior art salts necessary to arrive at the claimed 2-(2'-aminoethoxy) ethanol salt. *See Grabiak*, 769 F.2d at 731-32, 226 USPQ at 872 ("[I]n the case before us there must be adequate support in the prior art for the [prior art] ester/[claimed] thioester change in structure, in order to complete the PTO's *prima facie* case and shift the burden of going forward to the applicant."): *In re Lalu*, 747 F.2d 703, 705, 223 USPQ 1257, 1258 (Fed. Cir. 1984) ("The prior art must provide one of ordinary skill in the art the motivation to make the proposed molecular modifications needed to arrive at the claimed compound.")

Conclusion

We conclude that the PTO did not establish a *prima facie* case of obviousness, and thus did not shift to Jones the burden of coming forward with unexpected results or other objective evidence of non-obviousness. Accordingly, the decision of the Board is

REVERSED.

District Court, S.D. New York

Lipton v. The Nature Co.

No. 91 Civ. 3007 (RO)

Decided January 16, 1992

COPYRIGHTS

1. Elements of copyright — Statutory elements — Originality (§205.0707)

Plaintiff's compilation of terms of "venery," which are collective terms relating to nature and hunting, is copyrightable, although some terms are generally known and in public domain, since many terms were creatively interpreted from Middle English and therefore are products of plaintiff's imagination, and since arrangement of terms was original, reflecting plaintiff's consideration of factors such as fluidity of language and arrangement's lyrical and poetic potential.

2. Infringement pleading and practice — Jurisdiction (§217.05)

JUDICIAL PRACTICE AND PROCEDURE

Jurisdiction — Personal jurisdiction (§405.11)

Jurisdiction — Venue; transfer of action — In general (§405.1901)

Federal district court in New York has personal jurisdiction over non-resident who granted license to corporate defendant to manufacture and sell products on which are imprinted terms which allegedly infringe plaintiff's copyright, since claims arise from non-resident's contract to supply goods or services in New York, since claims allege commission by non-resident of tortious act outside New York which caused plaintiff injury within New York, and since non-resident should reasonably have expected that such action would have consequences within New York; venue in New York is proper, since copyright venue statute, 28 USC 1400(a), provides that venue is proper in district in which defendant "resides or may be found," and since defendant may be "found" in any district in which defendant is subject to personal jurisdiction.

Action by James Lipton against The Nature Co. and Michael Wein for copyright infringement. On defendants' motion to dismiss or to transfer. Motion denied.

Mark P. Ressler, of Kay Collyer & Boose, New York, N.Y., for plaintiff.

Noel M. Cook, of Owen Wickersham & Erickson, San Francisco, Calif.; Janet Dore, of Morgan & Finnegan, New York, and John P. Sutton, San Francisco, for defendants.

Owen, J.

Plaintiff James Lipton is an etymologist and author of the copyrighted book, *An Exaltation of Larks*, published originally in